IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Attorney Docket No. CPMC-010/00US

In re patent application of

SHANAZ H. DAIRKEE *et al.* Confirmation No.: 6434

Serial No. 09/816,460 Group Art Unit: 1637

Filed: March 23, 2001 Examiner: Cynthia B. Wilder

For: PROGNOSTIC METHODS FOR BREAST CANCER

DECLARATION UNDER 37 CFR § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

I, Shanaz H. Dairkee, hereby declare:

1. I am a co-inventor of the captioned application. I have worked in the field of cancer genetics since 1976. I have published over 45 peer-reviewed papers in this field. My curriculum vitae is attached. (See Attachment A).

2. I have read and understand the Office Action dated October 1, 2004, and particularly the Examiner's comments on pages 3 to 6, regarding the alleged non-enablement of claims 8-17 directed to a method for determining the likelihood of tumor reoccurrence in a patient or a method of identifying a patient as being at risk for breast cancer by analyzing a tissue cell sample for the level of expression of thyroid hormone receptor beta 1 (TRß1) gene, in the above-identified application.

3. In response to the Examiner's rejection, I herewith provide observations in support of applicants' position argued in the attached response that claims 8-17 are enabled by the specification of the above-identified application, which provides sufficient information so that a person skilled in the art can practice the present invention.

4. As noted by the Examiner, the present specification supports that the TRß1 gene is present at the chromosomal locus 3p24.3. (See page 5 of the October 1, 2004 office action). It is further noted that the Examiner acknowledges that the present specification on page 10 discloses

that the loss of heterozygosity (LOH) in morphologically normal cells is correlated with an increased risk for the development of cancer in those cells, and that the LOH at 3p24.3 is correlated with a decrease in expression of TRß1 in these cells. Beginning on page 19 of the present specification, disclosure is provided for the expression analysis of the TRß1 gene.

In an effort to eliminate any misconception that the Examiner may have had regarding the data provided in the specification, I wish to clarify specific points that the Examiner has characterized as being speculative. For example, the specification on page 20, lines 14-15, discloses transcript levels in normal breast organoids from healthy individuals undergoing reduction mammoplasty (not normal terminal ductal lobular units (TDLUs) of cancer patients) prior to cell culture and in cultures of the same specimen at passage 2 that showed close similarity. The Examiner further notes that breast cancer cell lines displayed a range of TRß1 gene expression. While some cell lines may be heterogeneous in terms of gene inactivation, i.e., they have a percentage of cells with TRß1 inactivation, this heterogeneity does not translate into (a) the data not being useful or (b) the data not being speculative. Additionally, the Examiner comments that only 30% (3/10) breast cancer cell lines showed lower to undetectable levels of TRß1 transcript. The present application does not propose that TRß1 gene inactivation is the only pathway to breast tumorigenesis, but rather, the present application provides a method that identifies a subset of breast tumors that may be triggered by this pathway and that some of the tested cell lines may reflect that subset.

5. In response to the Examiner's statement that the methylation data in the specification is inconclusive, the Examiner's attention is directed to the data provided in Tables 5 and 6 of the specification.

(i) Data from the methylation analysis of 11 matched sets of tumor and/or peripheral tissue is summarized in Table 5 and demonstrates the following: (a) TRß1 promoter methylation is relatively common in examined breast cancer sites; (b) tumors with TRß1 LOH, which are already lacking one allele, may display inactivation of the remaining one or more alleles by DNA methylation to escape the potential growth suppressing effects of this gene as evidenced in several instances where tumors do indeed display lack of nuclear receptor protein; and (c) in 5 cases, methylation was also displayed in the DNA of non-malignant peripheral tissue, which

suggests that in some normal TDLU partial or complete gene inactivation may involve DNA methylation.

(ii) In regard to Table 6 in the specification, 10 publicly available breast cancer cell lines and normal breast tissue derived cell culture were tested for the presence of TRß1 transcripts using RT-PCR. Two of the three breast cancer cell lines that were negative for TRß1 transcripts and analyzed for methylation of the TRß1 promoter tested positive for methylation using both MSP and COBRA methods. This result was reversed by 5-azaC, a demethylating agent, to obtain TRß1 gene expression, which validated that the TRß1 promoter was methylated. As a control, the data also shows that 3 of the breast cancer cell lines (BT474, CAMA1 and T47D) that were positive for TRß1 transcripts did not show methylation of the TRß1 promoter.

6. It is my opinion that the specification of the above-identified application provides the person skilled in the art with a method to analyze the expression of the TRß1 gene using techniques disclosed therein or other techniques that are well known to persons skilled in the art. See specifically paragraphs 0026 – 0033 of the above-referenced patent application which disclose testing a target cell sample from the periphery of carcinoma cells of the breast tumor and analyzing the methylation state of the chromosome in the region of the TRß1 promoter or analyzing the level of expression of TRß1 and determining which samples have lower TRß1 expression levels as compared to the control sample from the same patient (see page 12, lines 2-11). It is my opinion that undue experimentation is not required to correlate this data with LOH data and outcome of prognosis for breast cancer reoccurrence. The present specification guides the skilled person to perform the steps of the methods in claims 8-17 without any undue experimentation.

7. In support of providing additional data to the information provided in above-referenced patent application for using the parameter of the analysis of methylation of the TRß1 promoter to study the presence of TRß1 transcripts, I provide a copy of a post-published paper, of which my co-inventor, Dr. Li and I are co-authors with several other scientists, (See Attachment B), entitled "Biallelic Inactivation of the Thyroid Hormone Receptor ß1 Gene in Early Stage Breast Cancer." This paper reports the results of the analysis of TRß1 transcripts in breast cancer tumor cell lines and primary breast tumors and demonstrates a correlation between TRß1 promoter hypermethylation and

reduction of TRß1 transcripts in breast cancer cells. The data provided in this paper (particularly to page 1941, 2nd column to page 1942, 1st column and Fig. 2 of Attachment B) show that TRß1 methylation occurred in all 11 tested breast tumors using MSP and COBRA. As a control, 7 of 7 normal tissue from women without breast cancer were found to have no methylation. To evaluate concordance between TRß1 promoter methylation, LOH and expression of nuclear protein, tumor sections of patient samples where TRß1 methylation and allelic loss were previously confirmed in 7 cases. See Fig. 2(c), which shows the status of TRß1 promoter methylation and immunolocalization of gene product in primary breast cancer tissue. Attachment B confirms that TRß1 inactivation does indeed occur in a subset of primary breast cancer. Moreover, similar to the finding of LOH at 3p24.3 in normal TDLU, this data shows that the epigenetic alteration of TRß1 can also occur in nonmalignant tissue peripheral to carcinoma.

8. To verify that an assay measuring TRß1 RNA transcripts provide reliable data, I herewith provide the results of a robust method to measure the variation in TRß1 RNA transcript levels in non-malignant or malignant breast epithelial cells. Figure 1 (Attachment C) demonstrates the effect of the induction of the overexpression of the TRß1 gene in ENUt7 (a non-malignant breast cell line that has been developed as a model closely analogous to the genetically altered normal TDLU of cancerous breast tissue for studying early genetic deletions), and 3 malignant breast cancer cell lines (SKBR2, MDA435 and MCF7) also used in Table 6 of the above-identified application and in Attachment B. A publication entitled "Genome-wide Allelotyping of a New in Vitro Model System Reveals Early Events in Breast Cancer Progression" describes the properties of ENUt7 and the method by which this cell was produced is enclosed as Attachment D. The results show that the levels of TRß1 transcripts are increased in the "T" cells containing a vector containing the TRß1 gene over the endogenous TRß1 level in the "C" control cells that do not contain the vector containing the TRß1 gene. As evident by the consistently high level of GAPDH, a housekeeping gene, in all cell samples displayed, these results also show that RNA integrity is intact in the cells.

9. Figure 2 (Attachment E) is enclosed to show the negative effects of higher expression levels of TRß1 on malignant breast cancer cell lines, SKBR3 and MDA435, as compared to the same cells without a vector containing the TRß1 gene. The most dramatic negative effect of

TRß1 is observed on SKBR3 and MDA435 cell lines, both of which are originally TRß1 deficient. In ENUt7, the TRß1 gene displays some growth inhibition but to a lesser extent than the growth inhibition of SKBR3 and MDA435 cells. The ENUt7 cell line has been developed as a live model system to study the preneoplastic cells that may surround the breast tumor cells in a subset of breast tumors. The MCF7 cell line does not display any growth inhibitory effects by the overexpression of TRß1, and most likely represents a tumor subset that is not reliant on the inactivation of the TRß1 gene for its development. This data supports that higher levels of TRß1 inhibits tumor cell growth in certain subsets of breast cancer. See Figure 2 for a further explanation of the results.

10. Figure 3 (Attachment F) is enclosed to show the cellular blockade underlying growth inhibition at higher expression levels of TRß1 on the non-malignant cell line, ENUt7, as compared to the same cell without a vector containing the TRß1 gene. Panel A shows that the majority of cells (61.84%) are in S phase in the ENUt7 control cell without the vector containing the TRß1 gene, which means that the cells are going well. However Panel B shows that the ENUt7 cells containing the vector containing the TRß1 gene has only 24.31% of the cells in S phase and a marked increase in the number of cells in G1 phase (64.51%) as compared to ENUt7 cells without the TRß1 gene (38.16%). This data shows that increased levels of TRß1 are inhibitory to the growth of non-malignant cells that have been developed as a model to mimic the non-malignant cells around the periphery of a breast tumor. This data supports the proposition that higher levels of TRß1 inhibit cell growth in certain subsets of breast cancer. See Figure 3 for a further explanation of the results.

11. Thus, the data presented herein support the position that lower levels of TRß1 transcripts are correlated with LOH, and promoter methylation, and that higher levels of TRß1 inhibit the growth of certain sub-populations of tumor cells as well as non-malignant cells that surround the breast tumor cells and which appear to be normal. In conjunction with our data on 3pLOH in normal TDLU and the increased risk of recurrence, it appears likely that partial or complete TRß1 inactivation in normal TDLU, which results in a lower level of TRß1 transcript, enables the selection of cells in breast tissue, which are prone to the development of a new primary

tumor. Thus the measurement of relative levels of TRß1 transcript or gene product in normal TDLU indicates the risk of tumor recurrence in a certain subset of breast cancers.

12. I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____ Date: March 1, 2005

Shanaz H. Dairkee, Ph.D.

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SHANAZ HASHMI DAIRKEE, PH.D.
Curriculum Vitae

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EDUCATION:

1976	Columbia University, New York, Ph.D. *(Human Genetics and Development)*
1976-1981	Postdoctoral Fellow with Nobel Laureate Dr. Donald Glaser, Department of Molecular Biology, University of California, Berkeley, CA
Fall 2002	Course in Bioinformatics (UC Santa Cruz Extension)
2004	Certificate in Bioentrepreneurship (UC Berkeley, Haas School of Business)

PROFESSIONAL EXPERIENCE:

1981-1982	Assistant Research Geneticist, Dept. of Molecular Biology, University of California, Berkeley, CA
1982-1989	Staff, Peralta Cancer Research Institute, Oakland, CA
1990	Guest Scientist, Deutsches Krebsforchungzentrum, Heidelberg, Germany (Dr. Werner Franke's Lab)
1990-1994	Staff, Life Sciences Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA
1994-Present	Staff, Geraldine Brush Cancer Research Institute, California Pacific Med. Center, San Francisco, CA

SCIENTIFIC ORGANIZATIONS:

American Association for Cancer Research
New York Academy of Sciences
Tissue Culture Association

FUNDED SUPPORT AT CPMC:

1994-1998	Principal Investigator, "Selective Isolation of Transformed Breast Epithelium". NIH-1 RO1 CA66998. Direct Costs/yr: $120K, 30% Effort
1994-2001	Core Leader, Cell Biology Core, NIH 2 PO1 CA44768-10A1. "Molecular/Cellular Predictors of Breast Cancer Prognosis". (PI, H.S.Smith/F. Waldman). Direct Costs/yr: $95K, 30% Effort

1995-2002 Core Leader, Tissue Core Subcontract, NIH - CA58207 SPORE "Bay Area Breast Cancer Translational Research Program". (PI, J. W. Gray). Direct Costs, 2001-2002: $100K, 10% Effort

1997-2002 Principal Investigator, Project 2 Subcontract, "Molecular Markers Defining Women at High Risk for Breast Cancer", NIH - CA58207 SPORE "Bay Area Breast Cancer Translational Research Program" (PI, J .W. Gray). Direct Costs, 2001-2002: $100K, 30% Effort

1997-2000 Co Principal Investigator, "Micro-Quantity cDNA Libraries: Breast Tumor Expression", NIH - CA97012. (PI, K. W. Beisel). Direct Costs, entire period: $133K, 5-15% Effort

1998-2001 Principal Investigator, "Molecular Mapping of Surgical Margins", California Breast Cancer Research Program. Direct Costs/yr: $100K, 20% Effort

2000-2003 Principal Investigator, "Genetic Changes in Normal Epithelium of the Cancerous Breast", California Breast Cancer Research Program. Direct Costs, 2001-2002: $134K, 25% Effort

2001-2002 Principal Investigator, NSABP Award for Technology Development. Direct Costs, 2001-2002: $40K, 10% Effort

2002-2004 Principal Investigator, "Infinite Expansion of Breast Tumor Samples in Culture", California Breast Cancer Research Program. Direct Costs, 2003-2004: $100K, 15% Effort

2004-2009 Core Leader, Tissue Core UCSF Subcontract, NIH - CA58207 SPORE "Bay Area Breast Cancer Translational Research Program" (PI, J. W. Gray). Direct Costs, 2004-2005: $70K, 10% Effort

2004-2006 Co Investigator, NIH R01 UCSF Subcontract "High Resolution Genomic Analysis of Amplicon Structure" (PI, D. A. Albertson). Direct Costs, 2004-2005: $38K, 10% Effort

2004-2005 Co Investigator, DOD Concept Award (PI, G. Yount) "Novel Migration Behavior of Breast Cancer Cells May Predict Metastatic Potential". Direct Costs. $75K, 5% Effort

Pending

2004-2009 Principal Investigator, "Functional Analysis of Histologic Grade in Breast Cancer", NIH. Direct Costs, 2004-2005: $250K, 25% Effort

PROFESSIONAL SERVICE:

Reviewer, Cell Biology Study Section, U.S. Army Breast Cancer Research Program.
Ad Hoc Reviewer, NIH-NCI Innovative Small Grant Program
Ad Hoc Reviewer, USAMRMC Breast Cancer Research Program.
Reviewer, U.S. Department of Energy OCTR Laboratory Technology Research Program

Tissue Utilization Committee, Breast Oncology Program, UCSF. 1997-present.
Steering Committee, Bay Area SPORE in Breast Cancer. 1997-present.
Mentor, UCSF SPORE Career Development Program. 2002-present.
International Advisory Board, Panjwani Center for Molecular Medicine and Drug Research, 2003-present.

PATENTS:

U.S. Patent #4,853,464 - "Anti-Carcinoembryonic Antigen Antibodies", with Xoma Corporation

U.S. Patent #5,158,893 - "Methods and Compositions for Screening Carcinomas", with A.J. Hackett

U.S. Patent "Methods for cancer diagnosis and prognosis; screening methods for anticancer therapeutics". Filed.

PUBLICATIONS:

1. Dev, V.G., Miller, D.A., **Hashmi, S.**, Warburton, D., Miller, O.J., and Klein J. The biarmed *Mus poschiavinus* chromosome carrying the H-2 locus, Tlposklj is T7Bnr. *Genetics* 72:541-543, 1972.

2. **Hashmi, S.**, Allderdice, P.W., Klein, G., and Miller, O.J. Chromosomal heterogeneity in the RAG and MSWBS mouse tumor cell lines. *Cancer Research* 34:79-88, 1974.

3. Miller, D.A., Miller, O.J., Dev, V.G., **Hashmi, S.**, Tantravahi, H., Medrano, L., and Green, H. Human chromosome 19 carries a poliovirus receptor gene. *Cell* 1:167-173, 1974.

4. **Hashmi, S.**, May, S.R., Krooth, R.S., and Miller, O.J. Concurrent development of resistance to azauridine and adenosine in a mouse cell line. J. *Cell Physiol.* 86:191-200, 1975.

5. **Hashmi, S.**, and Miller, O.J. Further evidence of X-linkage of hypoxanthine phosphoribosyl-transferase in the mouse. *Cytogenet. and Cell Genet.* 17:35-41, 1976.

6. May, S.R., **Hashmi, S.**, Miller, O.J., and Krooth, R.S. Increased intracellular phosphoribosylphyrophosphate and accelerated orotic acid decarboxylation in a mouse cell line resistant to purine and pyrimidine ribonucleosides. *Somatic Cell Genet.* 3:263-279, 1977.

7. **Dairkee, S.H.**, and Gilbert, M.W. Production of factors required for cell attachment and spreading is a constitutive property in mouse A9 cells. *J. Cell Physiol.* 99:319-326, 1978.

8. **Dairkee, S.H.**, and Glaser, D.A. Dimethylsulfoxide affects colony morphology on agar and alters distribution of glycosaminoglycans and fibronectin. *Proc. Natl. Acad. Sci.,USA*, 79:6927-6931, 1982.

9. **Dairkee, S.H.**, and Glaser, D.A. A mutagen-testing assay based on heterogeneity in diameter and integrated optical density of mammalian cells. *Proc. Natl. Acad. Sci.,USA*, 81:2112-2116, 1984.

10. **Dairkee, S.H.**, and Glaser, D.A. Colony morphology on agar is an extremely sensitive indicator for growth effectors. *Exp. Cell Res.* 156:57-64, 1984.

11. **Dairkee**, S.H., Blayney, C.M., Asarnow, D., Smith, H.S., Hackett, A.J. Early expression of vimentin in human mammary cultures. *In Vitro Cellular &Dev. Biol.* 21:321-327, 1985.

12. **Dairkee, S.H.**, Blayney, C.M., Smith, H.S., and Hackett, A.J. Monoclonal antibody that defines human myoepithelium. *Proc. Natl. Acad. Sci.*, USA, 82:7409-7412, 1985.

13. **Dairkee, S.H.**, Blayney-Moore, C.M., Smith, H.S., and Hackett, A.J. Concurrent expression of basal and luminal epithelial markers in cultures of normal human breast analyzed with monoclonal antibodies. *Differentiation* 32:93-100, 1986.

14. **Dairkee, S.H.**, and Hackett, A.J. Potential for cytologic breast fluid analysis in breast disease. *Breast Cancer Res. &Treatment,* 8:21-23, 1986.

15. **Dairkee, S.H.**, Mayall, B.H., Smith, H.S., Hackett, A.J. Monoclonal marker that predicts early recurrence of breast cancer. *Lancet,* 1:514, 1987.

16. Smith, H.S., Wolman, S.R, **Dairkee, S.H.**, Hancock, M.C., Lippman, M., Leff, A., Hackett, A.J. Immortalization in culture occurs at a late stage in progression of breast cancer. *Journal of National Cancer Institute,* 78:611-615, 1987.

17. **Dairkee, S.H.**, Ljung, B.M., Smith, H.S., Hackett, A.J. Immunolocalization of a human basal epithelium-specific antikeratin in benign and malignant breast disease. *Breast Cancer Res. &Treatment,* 10:11-20, 1987.

18. Smith, H.S., **Dairkee, S.H.**, Ljung, B.M., Mayall, B., Sylvester, S.S., Hackett, A.J. Cellular manifestations of human breast cancer. *Cellular & Molecular Biol. of Experimental Mammary Cancer.* Ed. D. Medina, W. Kedwell, G. Heppner, E. Anderson, Plenum Co., New York, NY. 437-452, 1987.

19. Dardick, I., Rippstein, P., Skimming, L., Boivin, M., Parks, W.R., **Dairkee, S.H.** Immunohistochemistry and ultrastructure of myoepithelium and modified myoepithelium of the ducts of human major salivary glands: histogenetic implications for salivary gland tumors. *Oral med., Oral Surg., Oral Pathol.,* 64:703-715, 1987.

20. Dardick, I., Van Nostrand, A.W.P., Rippstein, P., Skimming, L., Hoppe, D., **Dairkee, S.H.** Characterization of epimyoepithelial islands in benign lympho epithelial lesions of major salivary gland: An immunohistochemical and ultrastructural study. *Head and Neck Surgery,* 10:168-178, 1988.

21. Bjorn, M.J., Smith, H.S., **Dairkee, S.H.** Response of primary human mammary tumor cell cultures to a monoclonal antibody-recombinant ricin A chain immunotoxin. *Cancer Immunol. Immunother.*, 26:121-124, 1988.

22. **Dairkee, S.H.**, Puett, L., Hackett, A.J. Expression of basal and luminal epithelium specific keratins in normal, benign and malignant breast tissue. *Journal of National Cancer Institute,* 80:691-695, 1988.

23. Dollbaum, C.M., Creasey, A.A., **Dairkee, S.H.**, Hiller, A.J., Rudolph, A.R., Lin, L., Vitt, C., Smith, H.S. Specificity of TNF toxicity for human mammary carcinomas relative to normal mammary epithelium and correlation with response to adriamycin. *Proc. Natl. Acad. Sci. USA,* 85:4740-4744, 1988.

24. **Dairkee, S.H.**, and Hackett, A.J. Internal antigens are accessible in breast cancer; implications for tumor targeting. *Journal of National Cancer Institute,* 80:1216-1220, 1988.

25. Dardick, I., Claude, A., Parks, W.R., Hoppe, D., Stinson, J., Burns, B.G., **Dairkee, S.H.** Warthin's Tumor: Ultrastructural and Immunohistochemical study of Basilar Epithelium. *Ultrastructural Pathology* 12:419-432, 1988.

26. Tsubura, A., Inui, T., Senkazi, H., Morii, S., **Dairkee, S.H.** Immunolocalization of the human basal epithelial marker monoclonal antibody 312C8-1 in normal tissue and mammary tumors of rodents. *Virchows Archiv. A. Pathol. Anat.* , 415:533-538, 1989.

27. **Dairkee, S.H.**, in *Cytometry.* Vol. 8, Number 3, Pg. 343,1989: *Immunocytochemical Technology* by Gwen V. Childs, Alan R. Liss, Inc., New York, NY., 1986. Book Review

28. Tsubura, A., Inui, T., Morii, S., **Dairkee, S.H.**, Oikawa, T., Matsuzawa, A. Loss of basal cell phenotype with acquisition of lung-colonizing capability in mouse mammary tumors. *Breast Cancer Res. &Treatment* 17:239-243, 1990.

29. **Dairkee, S.H.**, Puett, L., Counelis, A.M., Hackett, A.J. Accessibility to intracellular antigens within nutritionally-deprived human mammary epithelial cells. *Exp. Cell Res.* 192:182-188, 1991.

30. **Dairkee, S.H.**, and Hackett, A.J. Differential retention of Rhodamine 123 by breast carcinoma and normal human mammary tissue. *Breast Cancer Res. Treatment* 18:57-61, 1991.

31. Tsubura, A., Okada, H., Sasaki, M., **Dairkee, S.**, Morii, S. Immunohistochemical demonstration of keratins 8 and 14 in benign tumors of the skin appendage. *Virchows Archiv. A. Pathol. Anat.* , 418:503-507, 1991.

32. **Dairkee, S. H.**, and Heid, H. Cytokeratin profile of immunomagnetically separated epithelial subsets of the human mammary gland. *In Vitro Cell. and Dev. Biol.*, 29A:427-432, 1993.

33. Griffey, S.M., Madewell, B.R., **Dairkee, S.**, Hunt, J.E., Naydan, D.K., and Higgins, RJ. Immunohistochemical reactivity of basal and luminal epithelial-specific cytokeratin antibodies within the normal and neoplastic canine mammary gland. *Vet. Pathol.*, 30:155-161, 1993.

34. **Dairkee, S.H.**, Deng, G., Stampfer, M.R., Waldman, F.M., and Smith, H.S. Selective cell culture of primary breast carcinoma. *Cancer Research* 55:2516-2519, 1995.

35. **Dairkee, S.H.**, Kreis, C., Smith, H.S. Selective Isolation Of Transformed Breast Epithelium. *Radiation Oncology Investigations,* 3: 420-423, 1996.

36. **Dairkee, S.H.**, Smith, H.S. Genetic Analysis Of Breast Cancer Progression. *Journal of Mammary Gland Biology and Neoplasia,* Vol 1(2): 139-151, 1996. Invited Review

37. **Dairkee, S.H.**, Paulo, E.C., Traquina, P., Moore, D.H., Ljung, B-M., Smith, H.S. Partial Enzymatic Degradation of Stroma Allows Enrichment and Expansion of Primary Breast Tumor Cells. *Cancer Research,* 57:1590-1596, 1997.

38. **Dairkee, S.H.**, Smith, H.S. Biological Basis of Genetic Predisposition to Breast Cancer. *Advances in Oncobiology* 2: 137-158, 1998. Invited Review

39. Collins, C., Rommens, J.M., Kowbel, D., Godfrey, T., Tanner, M., Hwang, S., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, K.E., Froula, J., Cloutier, T., Kuo, W., Yaswen, P., **Dairkee, S.**, Giovanola, J., Hutchinson, G.B., Isola, J., Kallioniemi, O., Palazzolo, M., Martin, C., Erickson, C., Pinkel, D., Albertson, D., Li, W., Gray, J.W. Positional Cloning of *ZABC1* and *AIBC1*: Genes Amplified at 20q13.2 and Overexpressed in Breast Carcinomas. *Proc. Natl. Acad. Sci. USA* 95:8703-8708, 1998.

40. Chen, L-C., Manjeshwar, S., Lu, Y., Moore, D., Ljung, B-M., Kuo, W-L, **Dairkee, S.H**, Wernick, M., Collins, C., Smith, H.S. The Human Homolog for the C. elegans cul-4A Gene is Amplified and Overexpressed in Primary Breast Cancers. *Cancer Research* 58:3677-3683, 1998.

41. Pinkel, D., Segraves, R., Sudar, D., Clark, S., Poole, I., Collins, C., Kuo, W., Chen, C., Zhai, Y., **Dairkee, S.**, Ljung, B., Gray, J.W., Albertson, D.A. Comparative Genomic Hybridization to DNA Microarrays: High Resolution Analysis of Complex Patterns of Deletion and Amplification. *Nature Genetics,* 20: 207-211, 1998.

42. Li, Z., Bustos, V., Miner, J., Paulo, E., Meng, Z., Zlotnikov, G., Ljung, B-M., **Dairkee, S.H.** Propagation of Genetically Altered Tumor Cells Derived from Fine Needle Aspirates of Primary Breast Carcinoma. *Cancer Research,* 58: 5271-5274, 1998.

43. Albertson, D.G., Ylstra, B., Segraves, R., Collins, C., **Dairkee, S.H.**, Kowbel, D., Kuo, W-L., Gray, J.W., Pinkel, D. Quantitative Mapping of Amplicon Structure by Array CGH Identifies Vitamin D 24 Hydroxylase (CYP 24) as a Candidate Oncogene. *Nature Genetics,* 25: 144-146, 2000.

44. Li, Z., Moore, D.H., Meng, Z., Ljung, B-M., Gray, J.W., **Dairkee, S.H.** Increased Risk of Local Recurrence is Associated with Allelic Loss in Normal Lobules of Breast Cancer Patients. *Cancer Research,* 62: 1000-1003, 2002.

45. Li, Z., Meng, Z., Chandrasekharan, R., Kuo, W-L., Collins, C.C., Gray, J.W., **Dairkee, S.H.** Biallelic Inactivation of the Thyroid Hormone Receptor ß1 Gene in Early Stage Breast Cancer. *Cancer Research,* 62: 1939-1943, 2002.

46. Li, Z., Meng, Z., Sayeed, A., Shalaby, R., Ljung, B-M., **Dairkee, S.H.** Genome wide Alleotyping of a New *In Vitro* Model System Reveals Early Events in Breast Cancer Progression. *Cancer Research,* 62: 5980-5987, 2002.

47. Parmar, H., Young, P., Emerman, J.T., Neve, R.M., **Dairkee, S.H.**, Cunha, G.R. A novel method for growing human breast epithelium in vivo using mouse and human mammary fibroblasts. *Endocrinology,* 143:4886-4896, 2002.

48. Korkola, J. E., DeVries, S., Fridlyand, J., Hwang, E. S., Estep, A. L., Chen, Y. Y., Chew, K. L., **Dairkee, S. H.**, Jensen, R. M., Waldman, F. M. Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Research,* 63: 7167-7175, 2003.

49. **Dairkee, S.H.**, Ji, Y., Ben, Y., Moore, D.H., Meng, Z., Jeffrey, S.S. A molecular 'fingerprint' of primary breast cancer cultures; patterns resembling tumor tissue. *BMC Genomics,* 5: 47, 2004.

50. Meng, Z., Ben, Y., Li, Z., Chew, K., Ljung, B-M., Lagios, M.D., **Dairkee, S.H.** Breast cancer susceptibility gene aberrations occur early in sporadic breast tumors and in the acquisition of breast epithelial immortalization. *Genes Chromosomes and Cancer,* 41: 214-222, 2004.

51. Ylstra, B. Fung, M.Y., Meng, Z., Olshen, A., Massion, P.P., Jain, A.N., Ginzinger, D.G., Dairkee, S.H., Albertson, D.G. Coordinate Down Regulation of Phosphatidylinositol 3-Kinase and Vitamin D Receptor Expression in Primary Tumors. Submitted.

[CANCER RESEARCH 62, 1939–1943, April 1, 2002]

Advances in Brief

Biallelic Inactivation of the Thyroid Hormone Receptor β1 Gene in Early Stage Breast Cancer[1]

Zheng Li, Zhen Hang Meng, Raman Chandrasekaran, Wen-Lin Kuo, Colin C. Collins, Joe W. Gray, and Shanaz H. Dairkee[2]

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Abstract

Loss of heterozygosity within the short arm of chromosome 3 is a common molecular event in several types of solid tumors. In breast cancer, 3p loss of heterozygosity occurs in invasive tumor cells as well as in morphologically normal terminal ductal lobular units adjacent to carcinoma in some cases [G. Deng *et al.*, Science (Wash. DC), *274:* 2057–2059, 1996.]. The most frequent region of allelic loss at 3p24.3 in morphologically normal terminal ductal lobular units encompasses the thyroid hormone receptor β1 (*TRβ1*) gene. Here we have observed a variable degree of *TRβ1* promoter hypermethylation in all 11 cases of primary breast cancer examined. Moreover, hypermethylation occurred at the same CpG sites in nonmalignant tissue peripheral to carcinoma in 4 of 11 cases. The lack of *TRβ1* nuclear staining, a likely result of biallelic gene inactivation, was observed in 25% (22 of 85) of primary tumors. This is a first demonstration of promoter hypermethylation and a concurrent reduction of *TRβ1* transcripts in breast cancer cell lines, although specific CpG sites targeted for gene silencing remain to be determined. Gene expression was restored by treatment with 5-aza-deoxycytidine in such cases. The observation of early, frequent, and multiple mechanisms of *TRβ1* inactivation suggests a potential role for this gene in the suppression of breast tumorigenesis.

Introduction

Thyroid hormone receptors are ligand-mediated transcription factors, which form complexes with other nuclear receptors and multiple effector proteins to regulate growth, differentiation, and development (1). Loss of gene function associated with *v erb A*, a mutated variant of thyroid hormone receptor α (2) arrests normal differentiation of avian erythroblast progenitors resulting in virally induced leukemic transformation (3). Deletions encompassing *TRβ*[3] are suspected to play a role in the genesis of small cell lung cancer (4). In breast cancer, LOH is a common occurrence at chromosome 3p in the general vicinity of the *TRβ1* gene (5, 6). Most notably, LOH encompassing the *TRβ1* gene occurs before the manifestation of morphological changes in TDLUs of cancerous breast tissue (7, 8). We demonstrate here that epigenetic changes in the promoter region of *TRβ1* are also involved in the inactivation of this gene in breast cancer cell lines and possibly in early stage breast tumors.

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[3] The abbreviations used are: TRβ, thyroid hormone receptor β; LOH, loss of heterozygosity; TDLU, terminal ductal lobular unit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RARβ2, retinoic acid receptor β2; FISH, fluorescence *in situ* hybridization; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis; RT-PCR, reverse transcription-PCR.

Materials and Methods

RT-PCR Analysis. Established breast cancer cell lines and second passage normal mammary epithelial cells isolated from reduction mammoplasty tissue were propagated under routine culture conditions. Total cellular RNA was isolated from cells in the logarithmic growth phase using the RNeasy Mini kit (Qiagen). Up to 2 μg of total RNA was reverse transcribed using Superscript II RT RNase H- Reverse Transcriptase (Life Technologies, Inc.) and random hexamers (Operon, Inc.) in a reaction volume of 50 μl. In subsequent PCR reactions, 2 μl of cDNA were amplified. To normalize the relative amount of the cDNA synthesized for each test transcript, GAPDH was used as an internal competitive control within the same PCR reaction as the genes of interest. Gels were scanned, and the ratio of test gene:*GAPDH* was determined using ImageQuant (Molecular Dynamics). The PCR conditions were as follows: 94°C for 4 min 30 s to 30–35 PCR cycles at 94°C, 15 s; 58–61°C, 15 s; 72°C, 30 s; and a final 5 min extension at 72°C. PCR products were resolved in 2.0% agarose gel stained with ethidium bromide. Primer sequences were as follows:

GAPDH-F; TGATGACATCAAGAAGGTGGTGAA;
GAPDH-R: TCCTTGGAGGCCATGTGGGCCAT; (250 bp product)
TRβ1-F: GAACAGTCGTCGCCACATCTC;
TRβ1-R: TGAGCTCCCATTCCTCGTC; (500 bp product)
RARβ2-F: AGAGTTTGATGGAGTTGGGTGGAC; and
RARβ2-R: GCTGGCAGAGTGAAGGGAAAGTTT (721 bp product).

For restoration of *TRβ1* expression, cell lines were incubated with medium supplemented with 1 μM 5-aza-2-deoxycytidine for 5 days.

FISH, LOH, and Immunoperoxidase Analysis. For FISH, nuclear signals generated from two color hybridization of FITC-conjugated chromosome 3 centromere probe and Cy3-conjugated *TRβ1* probe (prepared and validated in the Division of Cancer Genetics, University of California, San Francisco Cancer Center) were visualized and recorded from 50 to 100 cells of each test culture. The proportion of nuclei displaying fewer copies of orange-colored *TRβ1* signal in comparison with the green colored centromeric signal was determined. Before LOH analysis of tumors, control DNA from nonmalignant skin of the patient was used to determine informative status (presence of heterozygosity) at the *EABMD* and *EABH* loci within the *TRβ1* region. Tumor DNA was isolated from manually microdissected, H&E-stained paraffin sections. PCR conditions were as described in Deng *et al.* (7). Samples were scored as positive for LOH if ≥30% reduction was observed in the allelic ratio of tumor compared with control DNA. A mouse monoclonal antibody (J51) specific for human *TRβ1* (Santa Cruz Biotechnology Biotech. Inc.) was used at 1:50 dilution for immunoperoxidase staining of multitumor tissue array sections from 0.8 mm cores of paraffin-embedded samples of 106 stage I invasive ductal breast carcinoma (assembled at the University of California, San Francisco Cancer Center). Antibody binding to tissue sections was visualized with the ABC immunoperoxidase kit (Vector Labs). Normal breast tissue served as a positive control for nuclear signal specificity. Tumor samples, which displayed ≥10% nuclear positive cells in a ×10 microscope field, were recorded as *TRβ1* positive.

DNA Extraction and Bisulfite Treatment. Fresh tissue from 7 cases of reduction mammoplasty and frozen blocks from 12 cases of pathologically confirmed stage I and II ductal breast carcinoma, and nonmalignant peripheral tissue were collected at the California Pacific Medical Center, San Francisco, CA, under Institutional Review Board approved guidelines. Genomic DNA was isolated from tissues and cell lines by the standard method of proteinase K digestion and phenol-chloroform extraction. A

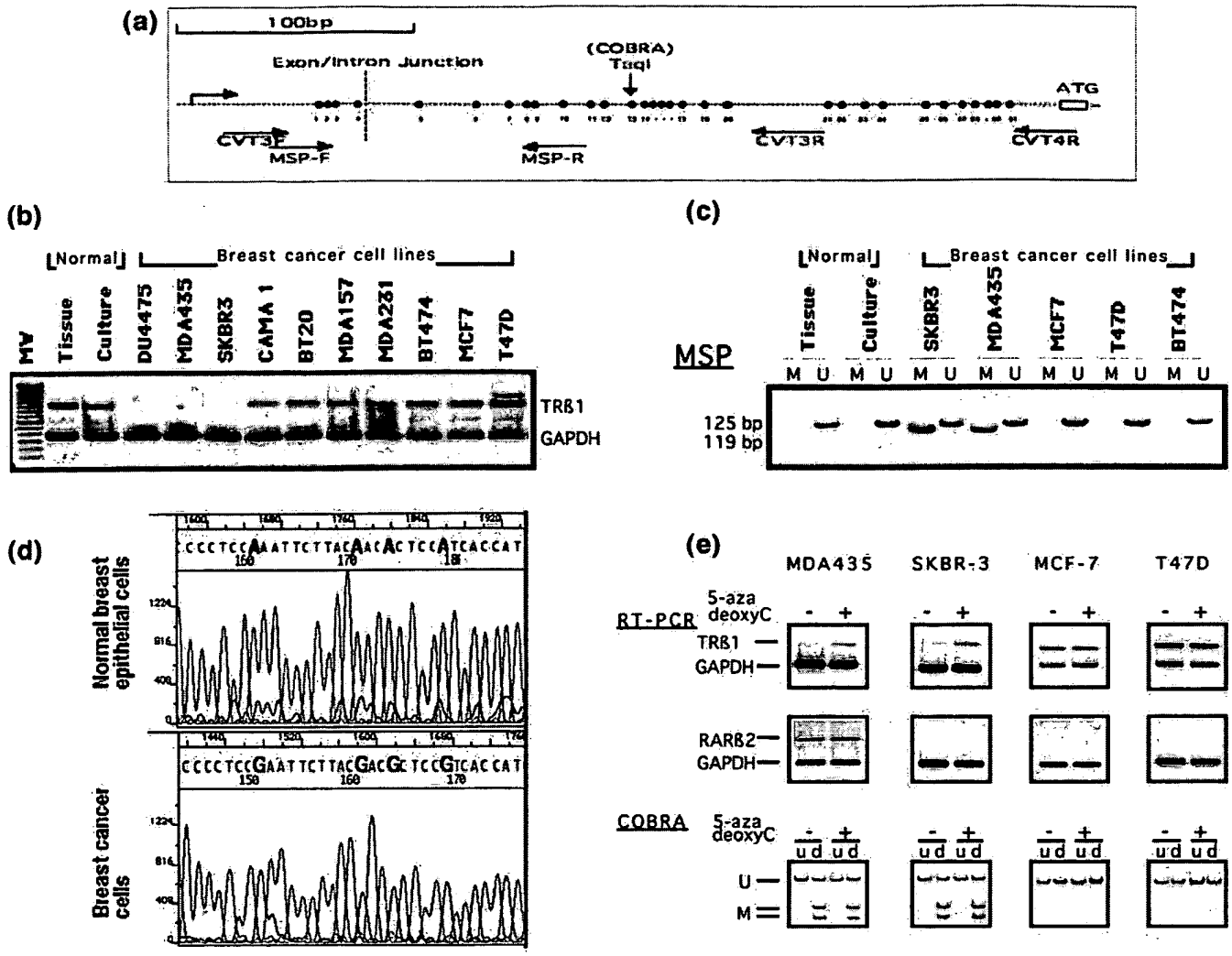

Fig. 1. TRβ1 expression and methylation analysis in breast cancer cell lines. *a*, genomic map of the CpG island in the 5′ promoter region of the *TRβ1* gene spanning the first exon and intron. ⌐→ represents the transcription initiation site. CpG sites are represented by *filled beads*. Within this 325 bp region, a total of 31 CpG sites are shown. *Arrows* represent PCR primer sets used for methylation analysis. *b*, relative expression of *TRβ1* transcript (500-bp PCR product) in normal and malignant breast epithelial mRNA. Corresponding level of *GAPDH* expression for each culture was measured simultaneously with *TRβ1* within the same PCR reaction mixture. Note that *TRβ1* levels in normal breast reduction mammoplasty tissue before cell culture *(Lane 2)* and cultures of the same specimen at passage 2 *(Lane 3)* show close similarity. *c*, MSP analysis of *TRβ1* promoter hypermethylation in breast cancer cell lines. The CVT3F/CVT3R primer set was used to amplify sodium bisulfite converted genomic DNA. The distinction between methylated and unmethylated alleles was based on amplification in the second round of a nested PCR with methylation-specific primers M1F and M1R or no methylation-specific primers, U1F and U1R. The product in *U* indicates the presence of unmethylated DNA, whereas the product in *M* indicates methylated DNA. *d*, bisulfite sequencing of *TRβ1* hypermethylated fragment using the CVT4R primer. *Top panel*, normal breast epithelial cells showing C→T conversion (unmethylated DNA). *Bottom panel*, breast cancer cell line MDA435 showing unconverted (methylated) CpG sites (indicated in *larger bolded font*). *e*, *TRβ1* expression before and after treatment with 1 μM 5-aza-2′-deoxycytidine in nonexpressing lines MDA435 and SKBR-3 compared with expressing lines MCF-7 and T47D. Note restoration of *TRβ1* expression in MDA435 and SKBR-3 cells. No change induced by drug treatment in MCF-7 and T47D cells. *Middle panels* demonstrate no effect of the demethylating agent on *RARβ* expression in the same mRNA samples shown in the *top panels*. Corresponding *GAPDH* and test gene expression levels were measured in the same PCR reaction mixture. *Bottom panels*, COBRA analysis on the cell populations shown in panels above. The products *U* and *M* indicate unmethylated and methylated DNA, respectively. *Lanes u* represent product before enzyme digestion. *Lanes d* represent cleavage of PCR products by TaqI, displayed as the two lower bands in MDA435 and SKBR-3 cells. Note concurrent reduction in the methylated DNA fraction of treated cells, which display *TRβ1* re-expression.

minimum of 10 ng (1000 cells) genomic DNA was treated with sodium bisulfite as described (9). On the basis of the *TRβ1* promoter sequence (Human Genome Project Working Draft),[4] a set of universal primers (CVT3F/CVT4R) was designed to amplify both the methylated and the unmethylated strands of bisulfite converted DNA. Additional analysis included MSP, and COBRA, as reported (9, 10). In the second round of the nested PCR assay, for MSP, primers M1F/M1R and U1F/U1R, and for COBRA (in tissue samples only), primers CVT3F/CVT3R were used. In COBRA, the PCR product was digested with TaqI to distinguish between methylated and unmethylated DNA. In each assay, absence of DNA template served as negative control, whereas the MDA435 cell line, where methylation was confirmed by sequence analysis of converted DNA, was used as a positive control.

MSP primers:

M1F: GGTAATTTGGTTAGAGGATCGCGC;

M1R: CACCCCTCCGATTCTTACGACG;

U1F: TATTGGTAATTTGGTTAGAGGATTGTGT; and

U1R: CACACCCCTCCAATT CTTACAACA

COBRA primers:

CVT3F: GTTTTAGGGTATTGGTAATTTGGT;

CVT4R: GACCACCCTATTCCACCACTA; and

CVT3R: CAAACTAATAACACCCCCACCA

The relative positions of the CpG sites covered by the above-mentioned primers are shown in Fig. 1*a*.

[4] Internet address: http://genome.ucsc.edu.

Results

***TRβ1* mRNA Expression and Gene Copy Number in Breast Cancer Cell Lines.** Toward a full understanding of the mechanisms of *TRβ1* inactivation during tumorigenesis, initially we analyzed relative gene expression in breast cancer cell lines by RT-PCR, using primers encompassing exons 4–7. Moderate *TRβ1* expression was observed in noncancerous breast epithelium before and after cell culture. Transcript levels below those of normal breast epithelial cells were observed in 7 of 10 cell lines (Fig. 1*b*; Table 1). Concurrently, we determined whether reduction in gene copy number was an underlying factor in the level of gene expression. In the majority of cell lines, FISH analysis with site-specific probes for interphase signal enumeration demonstrated equal numbers of chromosome 3 centromere and *TRβ1*-specific signals. In BT474 cells, reduction in copy number did not appear to influence expression level. In those cultures where gene expression was undetectable, such as MDA435 and SKBR3, fewer *TRβ1* gene copies were observed in the nuclei of a large proportion of cells (Table 1).

Homozygous deletions encompassing *TRβ1* were not found in any of 10 breast cancer cell lines examined. Similarly, in primary tumors, analyzed here (n = 12) and previously (64 stage I and II cases examined by LOH analysis, Ref. 8), chromosomal deletions were limited to loss of a single allele. To determine whether the remaining allele harbored inactivating gene mutations, cDNA of cell lines and primary tumors (12 cases) was analyzed by single-strand conformational polymorphism analysis of exons 9 and 10. This region consists of the ligand-binding domain, a common site of *TRβ1* germ-line mutations in syndromes unrelated to cancer (11). Because *TRβ1* mutations were not detected, here we have focused on the possible role of epigenetic mechanisms in gene inactivation.

Methylation of the *TRβ1* Promoter in Breast Cancer Cell Lines. On the basis of the presence of a CpG island in the 5′ region of the gene (Fig. 1*a*) we examined the role of promoter hypermethylation in *TRβ1* inactivation. Toward a rapid screen for *TRβ1* hypermethylation in noncancerous breast epithelial cells isolated from cosmetic reduction mammoplasty tissue and breast cancer cell lines, we first analyzed bisulfite converted DNA by the MSP assay (Fig. 1*c*). High gene expression levels were associated with the absence of DNA hypermethylation in MCF 7, T47D, and BT474 cell lines. Notably, a lack of detectable transcripts and promoter hypermethylation was concordant in MDA435 and SKBR3 cells. Subsequent sequence analysis of the MDA435 cell line demonstrated 27 of 27 potential sites in a 325-bp region within the CpG island of the gene promoter to be methylated. Four CpG sites downstream of the MSP region, where complete methylation was observed, are shown in Fig. 1*d*. Incomplete methylation evident at several sites (not shown) most likely accounts for the presence of unmethylated DNA in this cell line.

Table 1 *Status of TRβ1 copy number and relative gene expression in breast epithelial cultures*

Cell sample	FISH % nuclei with reduction in *TRβ1* signals[a]	RT-PCR Relative *TRβ1* intensity[b]
Nonmalignant		
Breast epithelium (tissue)	ND[c]	0.6
Breast epithelial culture (passage 2)	0.0	0.6
Breast cancer cell lines		
DU 4475	ND	0.0
MDA435	43.0	0.0
SKBR-3	37.0	0.0
CAMA 1	3.0	0.2
BT 20	7.0	0.4
MDA157	11.0	0.4
MDA231	15.0	0.5
BT 474	62.0	0.6
MCF-7	12.0	0.7
T47D	6.0	0.8

[a] Percent nuclei = (Nuclei where no. of signals of 3c > *TRβ1*)/(All nuclei evaluated) × 100.

[b] Relative *TRβ1* intensity = (Density of 500-bp *TRβ1* RT-PCR product)/(Density of 250 bp GAPDH RT-PCR product).

[c] ND, not done.

The detection of abnormally methylated sites in the promoter region is generally considered to be a strong indication of aberrant gene expression, although the target sites of methylation, which effectively inactivate the gene, may be far removed from the observed site of methylation. We have taken additional approaches to identify other methylation sites and additional links between methylation and lack of *TRβ1* expression. In methylation analysis by the COBRA method, the cell lines found to be methylated by MSP once again displayed hypermethylation (Fig. 1*e*).

Moreover, we have showed the restoration of *TRβ1* expression on treatment with a demethylating agent, 5-aza-deoxycytidine, demonstrating that silencing of the gene by this mechanism is partially reversible (Fig. 1*e*). We have observed a 43% and 20% reduction in DNA methylation status of 5-aza-deoxycytidine treated cultures of MDA435 and SKBR-3 cells, respectively. These results demonstrate a direct functional link between methylation and loss of expression, and demethylation and restoration of expression (Fig. 1*e*). Simultaneous analysis of another methylated gene in the 3p24 region, *RARβ2*, displayed discordant hypermethylation of the two genes in these samples suggesting that these events were not necessarily linked during tumor progression. Transcript levels of *RARβ2* were unaltered by treatment with 5-aza-deoxycytidine alone as reported previously (12, 13).

***TRβ1* Promoter Methylation, LOH, and Immunolocalization in Primary Breast Cancer.** In addition to breast cancer cell lines, we evaluated 11 matched cases of primary tumor and breast tissue peripheral to carcinoma for epigenetic alterations in the *TRβ1* promoter. We observed *TRβ1* hypermethylation in all 11 of the breast tumors concurrently by MSP and COBRA (Fig. 2, *a* and *b*). Considerable intertumor variability in the degree of methylation was evident by the intensity of the PCR products. In 7 of 7 independent cases of normal tissue from women without breast cancer, no methylation was detected by any of the methods used (representative examples shown in Fig. 1, *c* and *d*). Whereas it was surprising that the frequency with which *TRβ1* methylation occurred in established breast cancer cell lines was remarkably lower than tumor tissue, this finding may be related to years of *in vitro* selection. Methylation was not restricted only to the tumor cells but also occurred in nonmalignant tissue peripheral to carcinoma. In 4 of 11 cases, both CpG test sites in the promoter DNA were methylated. In the remaining 7 cases, no methylation was observed in the nonmalignant tissue or it was detected only within one of the two PCR-amplified regions of the CpG island. In one case (394T), in the quantitative COBRA assay, tumor DNA displayed less methylation than the peripheral tissue. Although the clonal relationship between the tumor and peripheral epithelium is not known in these samples, the *TRβ1* methylation data suggest the presence of heterogeneity in the nonmalignant component of the afflicted breast in this regard.

To evaluate the concordance between *TRβ1* promoter methylation, LOH, and expression of nuclear protein, we immunostained tumor sections of cases where *TRβ1* methylation and allelic loss was confirmed previously (n = 7). These cases exhibited complete loss of expression (4 cases) or a mixture of nuclear positive and negative tumor cells present within a single microscopic field (Fig. 2, *c, left panel*) indicating biallelic *TRβ1* silencing to be an emerging aberration in an evolving population of tumor cells.

To extend the analysis of aberrations in *TRβ1* gene expression to a

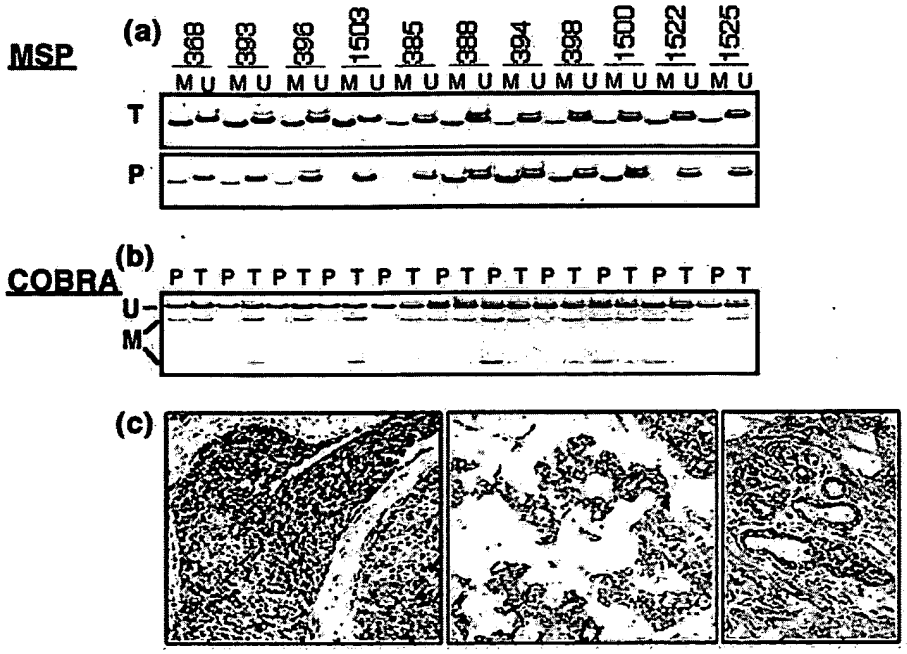

Fig. 2. TRβ1 promoter methylation and immunolocalization in primary breast cancer. *a*, MSP analysis of *TRβ1* promoter hypermethylation in primary breast tumor (*T*) and nonmalignant tissue peripheral to carcinoma (*P*). *M*, methylated DNA; *U*, unmethylated DNA. *b*, COBRA of primary breast tumor (*T*) and nonmalignant tissue peripheral to carcinoma (*P*). *c*, indirect immunoperoxidase staining of *TRβ1* in breast tumors visible as black nuclei. Nuclear fast red counter stain visible as *gray TRβ1*-negative nuclei. *Left panel*, strong nuclear staining of *TRβ1* in a proportion of tumor cells. *Middle panel*, primary tumor with no positively stained malignant cells showing nuclear counter stain only. *Right panel*, positively stained nuclei in normal TDLUs present within the *TRβ1*-negative tumor section shown in the middle panel. Magnification, ×100.

larger group of tumor samples, we evaluated tumor tissue arrays comprised of >100 cases of stage I primary breast carcinoma by immunoperoxidase localization of *TRβ1* gene product. Samples, which were uniformly negative for all of the cell types, were excluded based on the assumption of protein degradation. A lack of characteristic *TRβ1* nuclear immunostaining was observed in 18 of 78 cases (Fig. 2, *c, middle panel*). In these cases, nonmalignant constituents of the tumor sample, such as TDLU, lymphocytes, fibroblasts, and blood vessels continued to display *TRβ1*-positive nuclei (Fig. 2, *c, right panel*). The overall incidence of tumors lacking *TRβ1* nuclear expression in the patient subset studied here was 25% (22 of 85). At this time it remains unknown whether hypermethylation is involved in biallelic inactivation and gene silencing of all or a proportion of these cases.

Discussion

TRβ1 regulates gene expression when bound to thyroid response elements in the proximity of target genes (14). On the basis of the presence or absence of the ligand, thyroid hormone (triiodothyronine, T3), *TRβ1* can act as a transcriptional activator or silencer (15). *In vitro* studies have shown that T3 treatment of *TRβ1*-overexpressing cultures arrests proliferation in the G_0/G_1 phase of the cell cycle, and induces morphological and functional differentiation (16). Interestingly, T3-induced differentiation is preceded by a rapid decrease in the expression of the oncogene *c-myc* (17). Mechanisms, which inactivate *TRβ1*, may thus play an important role in the up-regulation of thyroid response elements harboring oncogenes during malignant progression. To our knowledge, this is the first direct demonstration of an epigenetic mechanism associated with *TRβ1* gene silencing in cancer.

Our analysis of the *TRβ1* gene in breast cancer cell lines provides evidence of promoter hypermethylation, decreased DNA copy number, and reversible reduction in transcript expression. *TRβ1* status of clinical tumor samples demonstrates that hypermethylation and LOH occur more frequently than the complete absence of nuclear protein. This suggests the possibility that the full extent of methylation required for gene inactivation was most likely under-represented because of the limited MSP and COBRA sites examined here. However, the detection of any abnormally methylated site is a strong indication that this mechanism could alter expression levels of the target gene. Nonexpressing tumors could serve as an important tool in mapping target CpG sites in the *TRβ1* promoter. A partially inactivating event, LOH in the region of the *TRβ1* gene, occurs before detectable morphological changes in normal TDLU adjacent to carcinoma (7, 8). Here, we have shown that the epigenetic alteration of *TRβ1* is also an early event, which occurs in nonmalignant tissue peripheral to carcinoma. Similar findings of estrogen receptor gene hypermethylation in normal colon tissue of cancer patients are postulated to be "field defects" (18).

Ligand-mediated prevention strategies for breast cancer have targeted *RARβ* (19), another epigenetically inactivated nuclear receptor superfamily member (12, 13). Our findings regarding multiple mechanisms of *TRβ1* inactivation support the importance of exploring this target as an additional approach for breast cancer control. However, a thorough understanding of the cellular consequences of modulating a ligand-induced master switch, such as *TRβ1*, is an essential prerequisite. Notably, the incidence of thyroid diseases, although controversial, is reportedly higher in breast cancer patients (20). An evaluation of thyroid function in the context of underlying breast biology in such cases could provide important clues regarding *TRβ1* as a potential factor that links disease manifestation in the two organ systems.

Acknowledgments

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References

1. Chen, J. D., and Evans, R. M. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature (Lond.), *377:* 454–457, 1995.
2. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., and Evans, R. M. The c-erb-A gene encodes a thyroid hormone receptor. Nature (Lond.), *324:* 641–646, 1986.
3. Kahn, P., Frykberg, L., Brady, C., Stanley, I., Beug, H., Vennstrom, B., and Graf, T. v-erbA cooperates with sarcoma oncogenes in leukemic cell transformation. Cell, *45:* 349–356, 1986.
4. Dobrovic, A., Houle, B., Belouchi, A., and Bradley, W. E. erbA-related sequence coding for DNA-binding hormone receptor localized to chromosome 3p21–3p25 and deleted in small cell lung carcinoma. Cancer Res., *48:* 682–685, 1988.

5. Ali, I. U., Lidereau, R., and Callahan, R. Presence of two members of c-erbA receptor gene family (c-erbA β and c-erbA2) in smallest region of somatic homozygosity on chromosome 3p21–p25 in human breast carcinoma. J. Natl. Cancer Inst., *81:* 1815–1820, 1989.
6. Matsumoto, S., Kasumi, F., Sakamoto, G., Onda, M., Nakamura, Y., and Emi, M. Detailed deletion mapping of chromosome arm 3p in breast cancers: a 2-cM region on 3p14.3–21.1 and a 5-cM region on 3p24.3–25.1 commonly deleted in tumors. Genes Chromosomes Cancer, *20:* 268–274, 1997.
7. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Wash. DC), *274:* 2057–2059, 1996.
8. Li, Z., Moore, D. H., Meng, Z., Ljung, B-M., Gray, J. W., and Dairkee, S. H. Increased risk of local recurrence is associated with allelic loss in normal lobules of breast cancer patients. Cancer Res., *62:* 1000–1003, 2002.
9. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.
10. Xiong, Z., and Laird, P. W. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res., *25:* 2532–2534, 1997.
11. Weiss, R. E., Weinberg, M., and Refetoff, S. Identical mutations in unrelated families with generalized resistance to thyroid hormone occur in cytosine-guanine-rich areas of the thyroid hormone receptor β gene. Analysis of 15 families. J. Clin. Investig., *91:* 2408–2415, 1993.
12. Sirchia, S. M., Ferguson, A. T., Sironi, E., Subramanyan, S., Orlandi, R., Sukumar, S., and Sacchi, N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor β2 promoter in breast cancer cells. Oncogene, *19:* 1556–1563, 2000.
13. Widschwendter, M., Berger, J., Hermann, M., Muller, H. M., Amberger, A., Zeschnigk, M., Widschwendter, A., Abendstein, B., Zeimet, A. G., Daxenbichler, G., and Marth, C. Methylation and silencing of the retinoic acid receptor-β2 gene in breast cancer. J. Natl. Cancer Inst., *92:* 826–832, 2000.
14. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., and Chambon, P. The nuclear receptor superfamily: the second decade. Cell, *83:* 835–839, 1995.
15. Damm, K., Thompson, C. C., and Evans, R. M. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature (Lond.), *339:* 593–597, 1989.
16. Lebel, J. M., Dussault, J. H., and Puymirat, J. Overexpression of the β 1 thyroid receptor induces differentiation in neuro-2a cells. Proc. Natl. Acad. Sci. USA, *91:* 2644–2648, 1994.
17. Perez-Juste, G., Garcia-Silva, S., and Aranda, A. An element in the region responsible for premature termination of transcription mediates repression of c-myc gene expression by thyroid hormone in neuroblastoma cells. J. Biol. Chem., *275:* 1307–1314, 2000.
18. Issa, J. P., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E., and Baylin, S. B. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat. Genet., *7:* 536–540, 1994.
19. Veronesi, U., De Palo, G., Marubini, E., Costa, A., Formelli, F., Mariani, L., Decensi, A., Camerini, T., Del Turco, M. R., Di Mauro, M. G., Muraca, M. G., Del Vecchio, M., Pinto, C., D'Aiuto, G., Boni, C., Campa, T., Magni, A., Miceli, R., Perloff, M., Malone, W. F., and Sporn, M. B. Randomized trial of fenretinide to prevent second breast malignancy in women with early breast cancer. J. Natl. Cancer Inst., *91:* 1847–1856, 1999.
20. Smyth, P. P. The thyroid and breast cancer: a significant association? Ann. Med., 29: 189–191, 1997.

hTRß1 expression in exogenously transduced breast epithelial cells

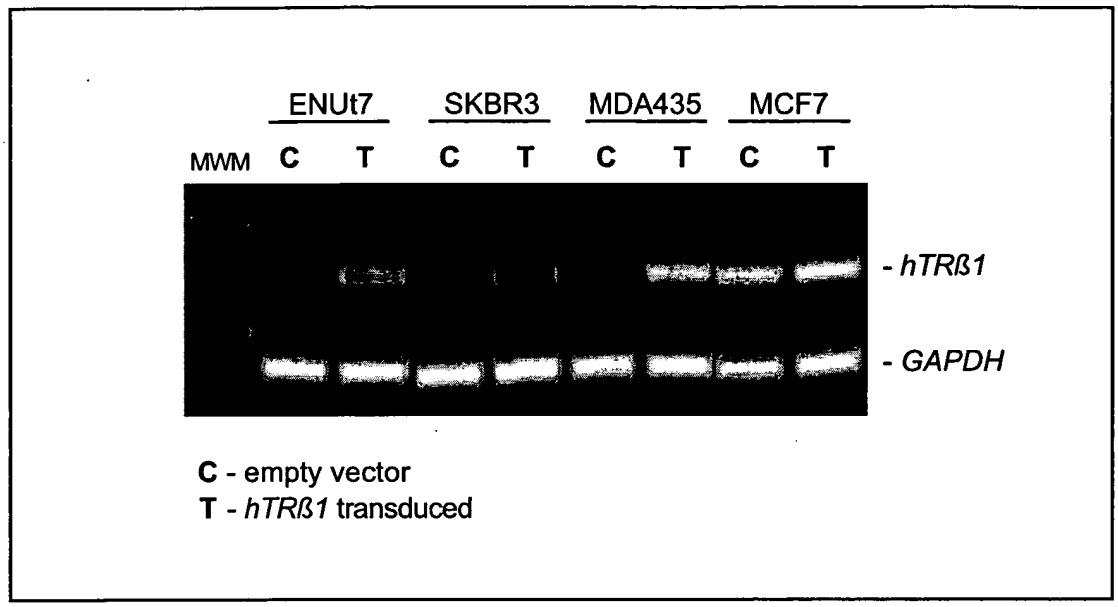

Figure 1 – Induction of gene overexpression in human breast epithelial cells transduced with *hTRβ1*. Lane T for ENUt7, SKBR3, and MDA435 cell lines display an observable relative increase in the *hTRβ1* transcript in comparison to the housekeeping gene, *GAPDH*. RNA was isolated using the Rneasy mini kit (Qiagen) and converted to cDNA with reverse transcriptase. The cDNA was subjected to PCR in a 25 microliter reaction mixture containing *hTRβ1* and *GAPDH* primers within the same reaction. PCR products were resolved on 2% agarose gels.

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Genome-wide Allelotyping of a New *in Vitro* Model System Reveals Early Events in Breast Cancer Progression[1]

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ABSTRACT

Toward the goal of identifying early genetic losses, which mediate the release of human breast epithelium from replicative suppression leading to cellular immortalization, we have used a newly developed *in vitro* model system. This system consists of epithelial cultures derived from noncancerous breast tissue, treated with the chemical carcinogen *N*-ethyl-*N*-nitrosourea, and continuously passaged to yield cell populations culminating in the immortal phenotype. Genome-wide allelotyping of early passage *N*-ethyl-*N*-nitrosourea-exposed cell populations revealed aberrations at >10% (18 of 169) loci examined. Allelic losses encompassing chromosomes 6q24-6q27, implicating immortalization-associated candidate genes, *hZAC* and *SEN6*, occurred in two independently derived cell lines before the Hayflick limit. Additional LOH sites were present in one cell line at 3p11–3p26, 11p15, and 20p12–13. Allelic losses reported in this cell line preceded detectable levels of telomerase activity and the occurrence of *p53*-related aberrations. Information gained from the search for early immortalization-associated genetic deletions in cultured cells was applied in a novel approach toward the analysis of morphologically normal terminal ductal lobular units microdissected from 20 cases of ductal carcinoma *in situ*. Notably, clonal allelic losses at chromosome 3p24 and 6q24 were an early occurrence in adjoining terminal ductal lobular units of a proportion of primary tumors, which displayed loss of heterozygosity (3 of 11 and 3 of 6, respectively). The biological insights provided by the new model system reported here strongly suggest that early allelic losses delineated in immortalized cultures and validated *in vivo* could serve as surrogate endpoints to assist in the identification and intervention of high-risk benign breast tissue, which sustains the potential for continuous proliferation.

INTRODUCTION

Cellular immortalization or infinite proliferative potential is a characteristic trait of cancer cells but occurs rarely in normal human cells. It is widely believed that the immortal phenotype is acquired through the selection of genetic and epigenetic alterations that activate proto-oncogenes and inactivate tumor suppressor genes during malignant progression. Although the full identity and precise sequence of cellular changes underlying immortalization is presently unknown, the widespread heterogeneity of neoplastic lesions of the human breast suggests that such changes may be acquired as stochastic events through multiple pathways (1). The application of surgical samples of pathological breast tissue for defining the sequence of key events in cellular immortalization is precluded by the inability to distinguish between defects conferring immortality and those related to other aspects of malignant transformation. Other limitations in defining temporal acquisition of the immortal phenotype include inaccessibility to precursor populations from the time of carcinogenic initiation through the course of malignant progression in the same patient. Thus, well-designed, biologically relevant cell culture models could facilitate a more pragmatic approach toward an in-depth understanding of the pathways to cellular immortalization.

Spontaneously immortalized breast cancer cell lines generally established from advanced malignant tumors have long played a critical role in functional experimentation. However, as they closely resemble tumor tissue in the above-mentioned limitations, these cell lines do not lend themselves well for defining causal changes underlying immortalization. Of more direct relevance to the acquisition of the immortal phenotype are spontaneously derived (2, 3) and carcinogen-induced cell lines (4), which have emerged from noncancerous breast epithelial cultures through continuous passaging. Experimental application of such cell lines and their derivatives have adequately established the usefulness of cell culture models for portraying well-defined genetic and phenotypic aspects characteristic of pathologically confirmed breast carcinoma (5–9).

In a reverse application, serially passaged cell populations could be effectively used for recapitulating the temporal sequence of immortalization-associated genetic changes in breast tissue from cancer patients. An important conceptual component of this strategy is the observation that specific genetic deletions commonly present in breast tumor cells are detectable in morphologically normal TDLUs[3] adjacent to carcinoma (10, 11). This finding implicates a proportion of TDLU in the cancerous breast as clonal precursors of malignant cells and proposes a new paradigm for identifying early aberrations related to malignant progression. However, direct searches of TDLU in this regard are severely hampered by qualitative and quantitative limitations of archived tissue. We have developed and used a new model system described here to assist in this search.

We report on the distinctive features of two novel human mammary epithelial cell lines immortalized with ENU and their application toward defining early genetic deletions, which may play a potentially causal role in cellular immortalization. First, by identifying specific molecular aberrations in continuously proliferating cells and subsequently validating their presence in pathological samples, we demonstrate that early passages of ENU-immortalized cell lines indeed have a counterpart in cancerous human breast tissue, which can be detected at the molecular level before the onset of proliferation-associated histological changes.

MATERIALS AND METHODS

ENU-Exposure and Cell Culture. Noncancerous breast tissue from a reduction mammoplasty procedure performed at the California Pacific Medical Center (San Francisco, CA) was obtained under Institutional Review Board-approved guidelines. For *in vitro* propagation of epithelial cells, organoids isolated from enzymatically dissociated tissue were plated in MM growth medium as described previously (12). The original primary culture initiated from organoids was designated as passage 1. Epithelial cultures at passage 2 were seeded at 5×10^4 cells/cm^2 into 10 independent 60-mm dishes for subsequent treatment and controls. Subconfluent cultures were treated twice within 7 days with 200 ng/ml ENU (Sigma). The cultures were not exposed to

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[3] The abbreviations used are: TDLU, terminal ductal lobular unit; ENU, *N*-ethyl-*N*-nitrosourea; TRF, terminal restriction fragment; RT-PCR, reverse transcription-PCR; DCIS, ductal carcinoma *in situ*; LOH, loss of heterozygosity; CK, cytokeratin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 *Chromosomal regions of allelic loss identified by genome-wide allelotyping analysis of self-selected early passage ENU-immortalized human breast epithelial cells*

Locus	Site	Presence of allelic loss				
		ENUt7-19′	ENUt7-28′	ENUt4A-31′	ENUt4A-44′	ENUt4J-31′
D3S2387	3p26.3	N[a]	Y	N	N	N
D3S4545	3p26.1	N	Y	N	N	N
D3S3038	3p22.3-23	N	Y	N	N	N
D3S2432	3p22.3	N	Y	N	N	N
D3S1766	3p21.1	N	Y	N	N	N
D3S4529	3p11.1	N	Y	N	N	N
F13A01	6p24.3-25.1	N	Y	N	Y	Y
D6S2439	6p22.1	N	Y	N	Y	Y
D6S1017	6p21.1	N	Y	N	Y	Y
D6S1053	6q11.2	N	Y	N	Y	N
GATA184A08[b]	6q24.2	Y	Y	N	Y	Y
D6S1277	6q26	N	Y	N	Y	Y
D6S1027[b]	6q27	Y	Y	N	Y	Y
D11S1984	11p15.5	N	Y	N	N	N
D11S1999	11p15.3	N	Y	N	N	N
D20S482	20p13	Y	Y	N	N	N
D20S604	20p12.1	N	Y	N	N	N
D20S481	20q13.12	N	Y	N	N	N

[a] N, no; Y, yes.
[b] These rows represent earliest allelic losses common to all ENU-exposed test populations.
′ indicates passage number.

any viral agents and were found to be free of *Mycoplasma* and human papilloma virus DNA sequences and SV40-large T antigen as determined by PCR assay and indirect immunostaining.

The epithelial origin of the cell lines was confirmed by indirect immunofluorescence of acetone-fixed monolayers seeded on microscope slides as previously described with mouse monoclonal antibodies to CKs 14 and 18 (13, 14).

Analysis of Immortalization-associated Allelic Loss. Initial PCR-based genotyping of ENU-treated populations was performed with 169 polymorphic markers comprising a low density Weber/RG set (details on markers are available on-line).[4] The mean heterozygosity index of the loci was 0.78. Twenty ng of DNA in a 10-μl PCR reaction mixture was amplified under the following conditions: denatured at 96°C for 30 cycles at 2 min each; denatured at 94°C for 45 s; annealed at 57°C for 45 s; extension at 72°C for 60 s; and final extension at 72°C for 7 min. Allelic size was determined with an ABI 377 DNA sequencer. Alterations in product size and/or allelic ratio were verified additionally in repeat PCR reactions with primers listed in Table 1. PCR products were resolved in 6–8% PAGE gels, visualized with Sybr-Green I (Molecular Probes) or with a silver stain kit (Pharmacia), scanned with Adobe PhotoShop, and quantitated by Image Quant. Allelic loss was defined as >50% reduction in the density of either allele compared with control DNA from untreated cells.

Determination of *p53* Status. Mutations in exons 4–9 of the *p53* gene were initially detected by PCR-single-strand conformational polymorphism analysis of cultures as described earlier (15) and confirmed by sequencing. For indirect immunostaining of paraffin-embedded tissue sections, anti-p53 mouse monoclonal antibody DO1 (Santa Cruz Biotechnology) at 1:100 dilution was used in conjunction with an ABC Immunoperoxidase kit (Vector Labs).

Telomerase Activity and Telomere Length Determination. Isolation of cell extract and the telomerase repeat amplification protocol was performed as per manufacturer recommendation (Trapeze Telomerase Detection Kit; Intergen). Briefly, the cell pellet was washed with PBS (Mg^{2+} and Ca^{2+} free), resuspended in 1× 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid lysis buffer at a concentration of 500 cells/μl, incubated on ice for 30 min, and centrifuged at 12,000 × *g* for 20 min at 4°C. PCR products were resolved by 10% PAGE and stained with Sybr Green-I (Molecular Probes). Heat-inactivated controls were included for each sample. A telomerase expressing positive control lysate was included in each test batch. Serial dilutions of lysates were tested to ensure that assays were in the linear range.

Genomic DNA was used to measure telomere lengths in an anti-digoxigenin alkaline phosphatase-mediated chemiluminescent assay based on Southern blotting and hybridization of TRFs with a digoxigenin-labeled probe specific for telomeric repeats (TeloTAGGG Assay; Roche Molecular Biochemicals). Control DNA samples representing known telomeric lengths of 3.9 kb (TeloLOW) and 10.2 kb (TeloHIGH) were run in parallel with the test samples. After exposure of the blot to X-ray film, an estimate of mean TRF length was obtained by visual comparison of the mean size of the smear in the test signal to the relative migration of molecular weight standards in 0.8% agarose gels.

RT-PCR Analysis. Total cellular RNA was isolated from cultured cells using RNeasy (Qiagen). Five hundred ng of total RNA were reverse transcribed with Superscript II Reverse Transcriptase (Life Technologies, Inc.) using random hexamers (Operon) in a reaction volume of 50 μl. PCR was performed using *GAPDH* as an internal control together with the test gene primers in a single 20-μl reaction volume. The upstream and downstream primer sequences were: *p16*, 5′-ATGGAGCCTTCGGCTGACTGG-3′ and 5′-GATCGGCCTCCGACCGTAAC-3′; *p21*, 5′-AGGATCCATGTCAGAACCGGCTGG-3′ and 5′-CAGGATCCTGTGGGCGGATTAGGGCT-3′; and *GAPDH*, 5′-TGATGACATCAAGAAGGTGGTGAA-3′ and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′.

PCR conditions included 94°C for 4 min 30 s; 94°C for 30 s; 64°C for 30 s; 72°C for 45 s; and a final 5-min extension at 72°C (30 PCR cycles). Products were resolved in 2.0% agarose gels and stained with ethidium bromide (Sigma). A semiquantitative determination of the level of *p16* and *p21* expression was made by comparing the density of the 123-bp *p16* or 510-bp *p21* product with the 250-bp *GAPDH* product.

Growth in Soft Agar. Cell suspensions containing ENU-immortalized cells in 0.3% agarose (Sigma) in growth medium were plated on top of a 0.7% agarose gel layer in 24-well plates at 10^4 cells/well. Cells were fed weekly with 200 μl of growth medium. After 3–4 weeks, anchorage-independent colonies consisting of ≥30 cells were counted. Established breast cancer cell lines, BT-474 and MCF7, used as positive controls, were plated at 100 cells/well and colony counts made at 5–10 days after plating.

Tumorigenicity. Six-to-8-week old female CB-17 SCID-beige mice (Taconic) were used for evaluating tumorigenic potential of ENU-immortalized cultures. Suspensions of 10^7 cells/100 μl were mixed in equal volume with 1 mg of Matrigel (Becton Dickinson) and injected into the left scapular region. Mice were housed in microisolator cages of a Thoren unit and allowed food and water *ad libitum*. Animals were inspected for tumor growth once weekly up to 118 days. Control mice bearing MCF-7 or BT-474 xenografts were euthanized at 40–45 days after cell inoculation because of tumor ulceration or where tumor burden interfered with the mobility of the animal.

Tissue Microdissection and LOH Analysis. Archival tumor blocks were obtained under Institutional Review Board-approved guidelines from 20 cases of pathologically confirmed high-grade DCIS treated at the California Pacific Medical Center. Formalin-fixed sections stained with methyl green or H&E were used for manual microdissection of tumor and morphologically normal TDLU adjacent to carcinoma using scalpel blades. For normal TDLU adjacent to tumor, cells of the same lobular unit were pooled from five serial sections. Samples containing 500-5000 cells were incubated in lysis buffer [10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 400 μg/ml proteinase K] at 50

[4] Internet address: www.marshmed.org/genetics/sets/scrset6.txt.

cells/μl for 24–96 h at 50°C. DNA template equivalent to 50 cells/sample was amplified for LOH studies. Because of artifacts commonly encountered in allelic representation and reproducibility of allelic ratios in limited DNA samples isolated from fixed tissue, duplicate parallel PCR reactions were run on each sample. Data were recorded only when variation in allelic ratio between duplicates was <5%. Nonmalignant skin epithelium or lymphocytes derived from uninvolved nodes were used for determination of the constitutional genotype. LOH calls were noted as described above.

RESULTS

Growth and Phenotype of ENU-immortalized Human Breast Epithelial Cell Lines

A reduction mammoplasty specimen, designated G385E, used for culturing normal breast epithelial cells toward subsequent carcinogen exposure and derivation of continuously proliferating cell lines, was obtained from a 23-year-old donor with no known breast pathology. To immortalize human breast epithelial cells, we used the direct acting carcinogen ENU, an alkylating agent known to induce rodent mammary tumors (16). A single concentration of 200 ng/ml ENU, applied twice within an interval of 7 days, was tolerated by primary breast epithelial cultures without apparent cytotoxicity. Unlike culture conditions reportedly important in the derivation of spontaneously immortalized cell lines, such as low calcium concentration (3) or the use of a chemically defined growth medium (2), we have used the MM growth medium in our experiments. The advantage of using the MM medium, also used in the derivation of benzo(*a*)pyrene-transformed cells (4), was that it only supports short-term growth (15–25 population doublings) of normal breast epithelial cells (12). Consequently, untreated control cultures were completely nonproliferative by passage 5. Spontaneous immortalization was not observed in the cells of this individual. In 6 of 8 culture dishes independently exposed to ENU, designated ENUt 3–8, a relatively low rate of mitotically active cells continued to occur after growth cessation in untreated controls. Two independent cultures, ENUt4 and ENUt7, were passaged continually and used in additional studies described below. Both populations were initially maintained at relatively high density (1:2 splits). As shown in Fig. 1, the cultures were slow growing up to passage 20 and displayed population-doubling times of 13–16 days. In the next 20 passages, the doubling time was reduced almost by 60%. The ENUt4 and ENUt7 cell lines are currently at passages 97 and 103, respectively, with doubling times of ~72 h. Clonal growth potential on solid substrate was tested and confirmed at passage 26 for both lines.

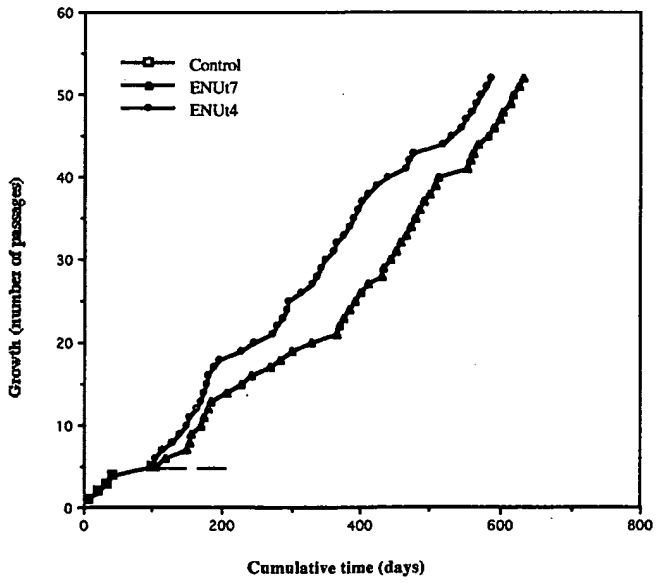

Fig. 1. Continuous growth of ENU-immortalized human breast epithelial lines, ENUt4 and ENU7. Untreated, control cultures displayed no growth after passage 5 (— — —).

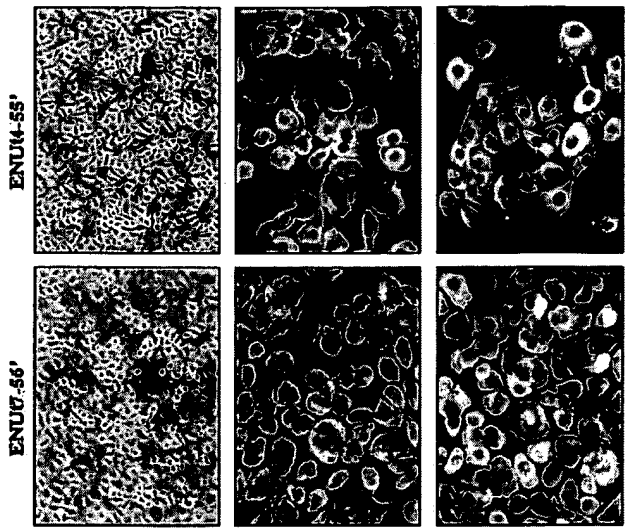

Fig. 2. Characteristic epithelial morphology of ENUt4 and ENUt7 cell lines (*left*: magnification ×40). Expression of CK 14 (*middle*: magnification ×400). Expression of CK 18 (*right*: magnification ×400). Passage number is indicated as '.

DNA polymorphism-based verification of authenticity of the novel cell lines, determined by allelotyping, demonstrated >90% concurrence with the constitutional genotype of the donor (partial data are shown in figure 4). The characteristic epithelial phenotype of the cell lines is illustrated in Fig. 2. As previously described by direct methods of CK analysis of breast epithelial cultures propagated in MM medium (17), concurrent expression of basal and luminal specific CKs was also observed here by indirect immunolocalization. In both ENUt4 and ENUt7 cell lines, CK 14 and CK 18 were abundantly expressed (Fig. 2).

Molecular Analysis of ENUt4 and ENUt7 Cell Lines

To capture the early events in the dynamic multistep process of ENU-induced carcinogenic progression leading to infinite growth potential, cell aliquots of early passages of proliferating cultures were cryopreserved for future evaluation. Subsequent analyses of continuously passaged cells are described below for two subclones of the ENUt4 cell line, ENUt4A and ENUt4J, maintained separately from passage 26, and for uncloned mass cultures of the ENUt7 line.

Telomerase Activity and Telomere Restriction Fragment Length. To determine the role of altered telomerase regulation in the acquisition of immortality as previously reported (18), we assayed for detectable enzymatic activity. No telomerase activity was detectable in untreated control cultures of human breast epithelial cells. In the ENUt7 cell line, telomerase was first apparent at passage 30 and continued to be maintained with increasing passages of this cell line. In contrast, as shown in Fig. 3*a*, both subclones of the ENUt4 cell line sustained growth without telomerase induction for 73 population doublings (passage 73). In the case of the ENUt4 cell line, the induction of telomerase did not appear to be critical until passage 88, when activity was first detected (data not shown).

Mean TRFs of ENUt7 and both subclones ENUt4A and ENUt4J, measured at passages 60–101, were between 2.5 and 4.5 kb (Fig. 3*b*). In comparison to the untreated control epithelial cells (average TRF length, 7.5 kb) the telomeres of the ENU-immortalized cultures were considerably shortened as reported for other immortalized human

mammary epithelial cells, which have emerged gradually through the immortal conversion process (8).

Early Allelic Losses. To establish an initial baseline of genetic deletions associated with the potential for continuous growth in culture, allelotyping was first performed on DNA isolated from ENUt7 cells at passages 12 and 28 with 169 polymorphic markers providing a genome-wide scan with an average spacing of 24.2 cM. No abnormalities were detectable at passage 12. Cells at passage 28 displayed deviation from a 1:1 constitutional allelic ratio at 23 polymorphic sites. Subsequent LOH analysis confirmed allelic loss at 18 loci (Table 1). The next phase of LOH analysis at these loci was extended to ENU-treated populations, cryopreserved before passage 28 or thereafter.

As summarized in Table 1, the first evidence of allelic loss in the ENUt7 cell line was observed as early as passage 19 (~19 population doublings) at loci *GATA184A08* and *D6S1027* on chromosome 6q24 and 6q27, respectively, and at *D20S482* on chromosome 20p13. LOH at additional loci on chromosomes 3p, 6p, 6q, 11p, 20p, and 20q occurred within the next 9 passages. Notably, the acquisition of LOH at loci on 6p and 6q was common to both ENU-immortalized cell lines, although in the ENUt4 cultures, LOH was first observed several passages after its appearance in ENUt7 (Fig. 4). Initially, the LOH profiles of the two subclones, ENUt4A and ENUt4J, appeared somewhat dissimilar. At passage 31, whereas ENUt4J displayed several regions of loss on chromosome 6, in ENUt4A, only a single partial deletion at 3q13 was apparent. This population was replaced with cells that displayed all of the losses observed in ENUt4J by passage 44. However, the two ENUt4 subclones continued to be distinct from each other at locus *D6S1053*, where LOH was observed only in ENUt4A. On the basis of the similarities in the early LOH profiles, we conclude that sites of allelic loss common to the ENUt4 and ENUt7 cell lines may be indicative of inactivated candidate genes critical for suppressing the initiation of the pathway to ENU-induced cellular immortalization.

p53, p21, and p16 Alterations. Allelotyping of early passage ENU-immortalized cells did not reveal genetic deletions in the region encompassing the *p53* tumor suppressor gene at chromosome 17p. We asked whether a mutated *p53* gene might have played a role in the

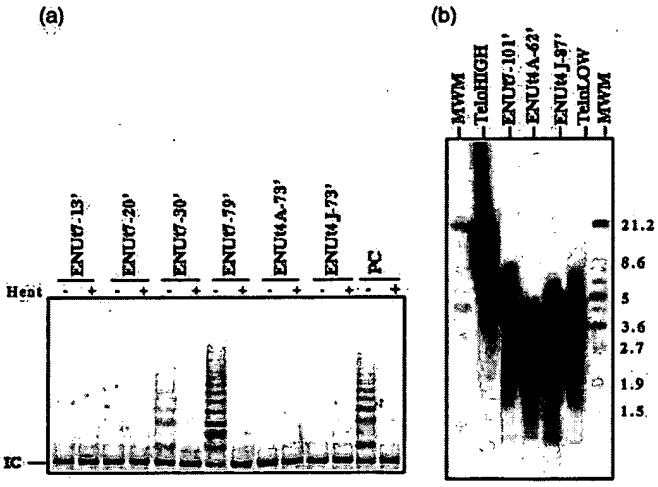

Fig. 3. *a*, telomerase activity in ENUt4 and ENUt7 cell lines. Extracts of 1000 cell equivalents were tested by the telomerase repeat amplification protocol assay; heat + indicates heat-treated controls. *IC* refers to an internal control to demonstrate the absence of PCR inhibitors in the cellular extract. *PC* (positive control) refers to telomerase activity in the breast cancer cell line T47D. *b*, TRF-based telomere length analysis in late passage ENUt cell populations. In all cases, telomeric chromosomal ends are considerably eroded but stabilized. TeloLOW and TeloHIGH are genomic DNA from control cell lines with a mean TRF length of 3.9 and 10.2 kb. MWM, molecular weight marker.

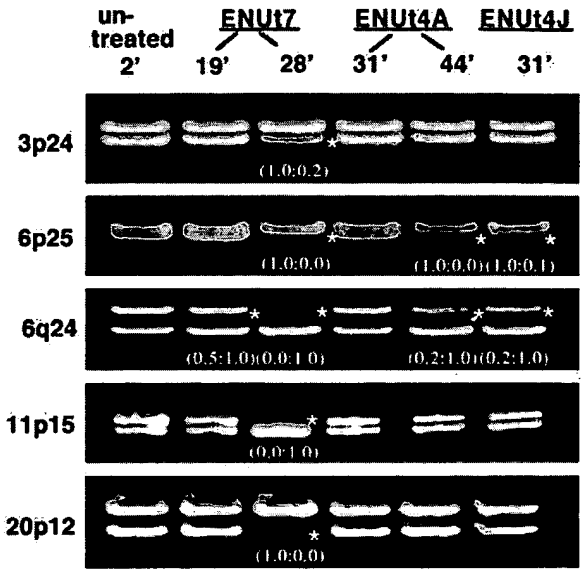

Fig. 4. PCR-based LOH analysis of DNA. Untreated control cells demonstrated a 1:1 allelic ratio at all loci tested. Earliest loss observed is partial LOH at chromosome 6q24 (locus *GATA 184A08*) in the ENUt7 cell line at passage 19. At passage 28, the culture shows complete LOH at this locus. Deletions at 3p24 (locus *D3S3038*), 6p25 (locus *F13A01*), 11p15 (locus *D11S1984*), and 20p12 (locus *D20S604*) were first observed at passage 28. In subclones ENU4A and ENUt4J, LOH was first detectable at passages 44 and 31 at 6p25 and 6q24, respectively. *Asterisks* denote allelic loss. Allelic ratio for each deleted locus is shown in *parentheses*.

acquisition of genome-wide LOH in these cell lines. In ENUt4A and ENUt4J cells, *p53* mutations have not been observed up to the most recent passage assayed (passage 63). In the ENUt7 cell line, *p53* mutations were observed only in a subset of cells at passage 28 when the entire culture homogeneously displayed complete allelic loss at chromosome 6q24-27. The occurrence of LOH at 6q24-27 thus appears to be *p53*-independent in these cells. At passage 31, the ENUt7 culture consisted of a mixed cell population with *p53* missense mutations within exon 7 at codon 239 and within exon 8 at codon 267. By passage 65, the cell line displayed mutations only within exon 7 (Fig. 5*a*).

Loss of expression of the *p53*-regulated *p21* gene, commonly encountered in breast cancer cell lines, was first detectable in ENUt7 cells at passage 74 (Fig. 5*b*). Similarly, for the *p16* gene, upstream of the *pRB* growth regulatory pathway, although relatively high transcript levels were detected up to passage 28, a visible reduction in gene expression was observed in the ENUt7 cell line after passage 31 (Fig. 5*c*). For both *p21* and *p16* genes, ENUt4 subclones continued to display high transcript levels at last evaluation (up to passage 74). Similar to the ENU-immortalized cell lines, heterogeneity in the loss of *p16* expression was also apparent in established breast cancer cell lines. For example, in MCF-7, T47D, and MDA 231 cells, *p16* transcripts were undetectable, whereas in SKBR3 and BT-474 cell lines, *p16* levels were relatively high (Fig. 5*c*).

Anchorage Independence and Tumorigenicity. With regard to the presence of classical transformation endpoints in ENU-immortalized cell lines, such as anchorage-independent growth and tumorigenicity in murine hosts, our analysis was limited to later passages. The cloning efficiency of ENUt4 (passage 66) and ENUt7 (passage 95) cells plated in 8–10 replicate wells in soft agar was 0.1–0.3%. Established breast cancer cell lines, MCF-7 and BT-474, displayed up to 100-fold greater cloning efficiencies (11 and 7%, respectively) under similar experimental conditions. Subsequent expansion and replating of ENUt4 and ENUt7 colonies picked from soft agar showed

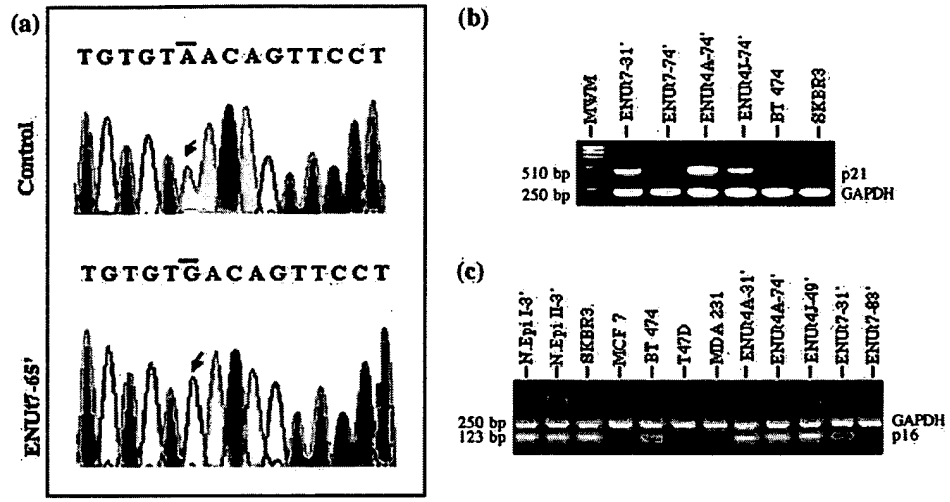

Fig. 5. Genetic alterations in ENU-immortalized human breast epithelial cells. *a, p53*: DNA sequence of late passage ENUt7 showing a homogeneous population of cells with a missense mutation from AAC to GAC at codon 239 of exon 7, resulting in the replacement of asparagine with aspartic acid. No mutations were detected in untreated control cells. *b*, detection of *p21* down-regulation in ENU-immortalized cells by semiquantitative RT-PCR analysis. Experimental conditions were optimized for coamplification of *p21* and *GAPDH* primers. Loss of *p21* expression observed in late passage ENUt7 but not in ENUt4A and 4J. A relatively high basal level of expression was maintained at earlier passages (data on early passage ENUt4A and 4J not shown). Breast cancer cell lines BT-474 and SKBR3 displayed reduction of *p21* expression similar to ENUt7–74'. MWM, molecular weight marker. *c*, RT-PCR analysis showing relative *p16* expression in normal breast epithelial cultures at passage 3 in two independent cases (*Lanes 1* and *2*), breast cancer cell lines (*Lanes 3–7*), and ENUt4 and ENUt7 populations (*Lanes 8–12*). Substantially lower *p16* expression is visible in three of five breast cancer cell lines. A gradual reduction in *p16* expression with increasing passage is seen only in the ENUt7 cell line.

relative increases in anchorage-independent cloning efficiency to 1 and 1.8%, respectively.

Inoculation of ENUt4 (passage 66) and ENUt7 (passage 95) cell lines into immune compromised mice ($n = 9$ for each cell line) did not yield measurable tumor growth up to 118 days of observation. Upon termination of the experiment, mice were euthanized, and the cell injection site was harvested and processed for histological analysis. No evidence of tumor formation was apparent at the microscopic level. In contrast, in independent experiments, palpable lesions within 10–15 days of inoculation and high tumor take were observed in the breast cancer cell lines MCF-7 ($n = 12$, 100% tumorigenicity) and BT-474 ($n = 24$, 92% tumorigenicity).

Contemporaneous Alterations in ENU-immortalized Cell Lines and Cancerous Breast Tissue

Allelic Losses. We asked whether allelic losses observed in early passage ENU-immortalized cells *in vitro* were contemporaneous with those *in vivo*. In other words, do such allelic losses also occur early during malignant progression? To address this, DNA was isolated from microdissected foci of preinvasive primary breast tumors classified as DCIS and compared with that of putative clonal progenitor cells, *i.e.*, morphologically normal adjacent TDLU in close proximity to the tumor (Fig. 6*A*). For evaluating the full spectrum of LOH-harboring loci observed in ENU-immortalized cells while DNA preparations from malignant sites were quantitatively adequate, the DNA yield of individual normal TDLU was a limiting factor. Therefore, LOH analysis of tissue-derived DNA was confined to two early candidate regions of deletion each on chromosome 3p and 6q. Nineteen of 20 DCIS cases examined were informative at one or more loci.

As summarized in Table 2, of the earliest deletions common to both ENU-immortalized cell lines, LOH at *GATA184A08* (6q24) was also observed in microdissected tumor cells of 6 of 11 informative cases. Notably, in 3 of 6 cases, LOH was displayed by morphologically normal adjacent TDLU as well (Fig. 6*B*). On the other hand, at locus *D6S1027* (6q27) whereas LOH occurred in the tumor cells of 4 of 8 informative cases, adjacent TDLU showed normal allelic ratios. At two loci encompassing 3p24 (*TRβ* and *D3S2423)*, 11 of 16 informative cases displayed allelic loss in the tumor cells. In 3 of 11 cases, LOH was also observed in morphologically normal TDLU (Fig. 6*C*). Deletions displayed by tumor/normal TDLU pairs were allele specific, providing strong supportive evidence for a clonal relationship between the two groups of microdissected cells. Of those DCIS cases

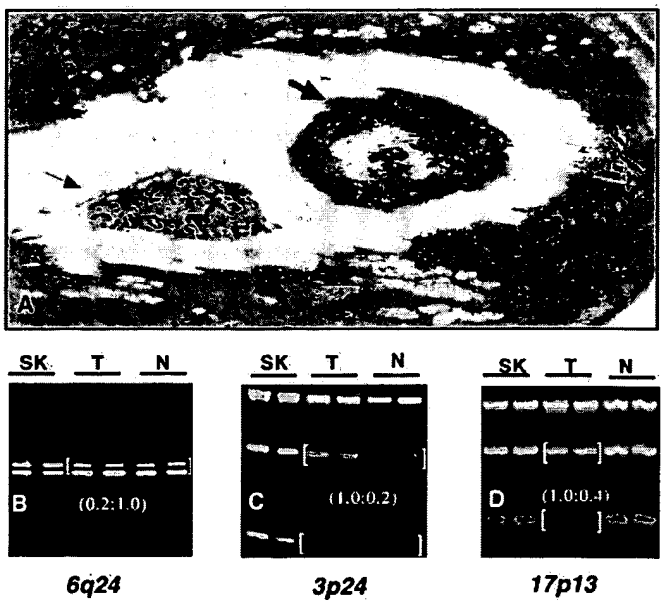

Fig. 6. Immortalization-associated LOH in microdissected populations from early stage breast tumor tissue. *A*, tissue surrounding the area of interest was scraped away to harvest highly enriched cell fractions from H&E-stained sections for LOH analysis. *Large arrow* indicates DCIS. *Small arrow* indicates a morphologically normal adjacent TDLU. For LOH analysis, each DNA sample was amplified in duplicate PCR runs and the products resolved within the same gel in parallel lanes to minimize experimental artifacts often observed with paraffin-embedded archival material. *Lanes SK* show 1:1 allelic ratio in uninvolved skin tissue of the patient. *Lanes T* denote microdissected tumor cells. *Lanes N* denote morphologically normal TDLU adjacent to carcinoma. The deleted allele is indicated within []. Altered allelic ratio is shown in parentheses. *B*, LOH displayed by tumor and normal TDLU at 6q24 (locus *GATA 184A08*). *C*, LOH displayed by tumor and normal TDLU at 3p24 (locus *TRβ*). *D*, LOH at 17p13 (locus *TP53.PCR8*) only in tumor DNA.

Table 2 *Pathological features and early genetic changes in breast tissue of patients diagnosed with DCIS*

Case	Patient age (yrs)	Tumor size (mm)	DCIS subtype	Comedo necrosis	Nuclear[a] *p53* in		LOH in	
					Tumor	TDLU	Tumor	TDLU
62	51	50	B[b]	Y[c]	N	N	Y (a)[d]	Y (a)
157	52	7	A	N	N	N	N	N
180	43	10	A	Y	N	N	Y (c)	N
351	52	20	A	Y	N	N	Y (a, c)	Y (a,c)
355	45	14	C	N	Y	N	Y (a, c)	Y (c)
499	47	10	B	Y	N	N	Y (c, d)	N
520	49	8	A	Y	N	N	N	N
524	42	na	A	N	N	N	N	N
583	76	12	A	N	N	N	Y (d)	N
625	56	12	A	Y	N	N	Y (b)	N
637	65	24	A	Y	N	N	Y (b)	N
752	70	45	C	Y	N	N	Y (b, c)	N
783	78	10	B	Y	N	N	Y (b)	N
788	71	19	A	Y	N	N	Y (a, b)	N
792	64	18	A	Y	Y	N	NI	
793	67	14	A	Y	Y	N	Y (c)	N
833	35	10	A	Y	N	N	Y (b, c, d)	Y (b,c)
852	75	19	A	Y	N	N	Y (b)	N
898	35	7	A	N	N	N	Y (a, b, d)	N
939	43	25	B	Y	N	N	N	N

[a] Presence of *p53* gene product was evaluated by immunohistochemistry.
[b] A, major category: solid; B, major category: micropapillary; C, major category: cribriform.
[c] Y, yes; N, no; na, not available; NI, noninformative.
[d] LOH sites indicated within (): 3p24: *TRβ* (a); *D3S2423* (b); 6q24: *GATA184AO8* (c); *D6S1027* (d).

where LOH was observed in morphologically normal TDLU in 2 patients, loss was either at 3p24 or at 6q24, whereas in another two cases, allelic loss was simultaneously displayed at both sites. No association was observed between the presence of LOH in TDLU and known clinical or pathologic features of the sample.

p53 Inactivation. Allelic loss encompassing the *p53* gene at 17p13 (loci *D17S1541* and *TP53.PCR5–8*) was common in the tumor cells and occurred in 12 of 18 informative cases. However, no deletions were found in normal adjacent TDLU (Fig. 6*D*). Similarly, immunopositive nuclei, indicative of a predominant class of *p53* mutations in cancer, were observed only in the malignant cells of 3 of 20 patients with these early stage tumors (Table 2). Nuclei of normal adjacent TDLU were negative for immunostaining in all cases. Thus, it appears that the occurrence of deletions or mutations, which inactivate the *p53* gene most likely are not an underlying factor in the accrual of allelic loss at chromosome 3p24 or 6q24 in morphologically normal TDLU adjacent to carcinoma.

DISCUSSION

Inactivation of growth-regulating genes involved in the immortalization of tumor cells occurs through multiple mechanisms, including loss-of-function mutation of one allele, presumably induced by a carcinogenic DNA insult, often accompanied by LOH. Genome-wide analyses of tumor tissue have demonstrated that allelic loss is common in breast cancer but occurrence sites vary widely between tumors (19–21). Sites of allelic loss shared by invasive tumor cells and accompanying preinvasive, or premalignant foci, such as DCIS and atypical hyperplasia, respectively, have suggested a clonal relationship between these microscopically distinct lesions (22) but have not revealed the sequence of acquisition of tumor-associated allelic losses conferring cellular immortality. Here, we have applied ENU-immortalized human mammary epithelial cells toward delineating the temporal relationship between early genomic deletions and the acquisition of known cellular alterations leading to infinite growth potential *in vitro* and in cancerous tissue.

Previous reports on the induction of immortalization in normal human mammary epithelial cells without viral oncogenes have demonstrated that functional telomerase and inactivation of the pRB/p16^{INK4} pathway are obligatory steps (9, 18). With regard to these and other known growth regulatory changes associated with immortalization, our data derived from the analysis of the ENUt7 cell line are in agreement. For example, at relatively early passages, the ENUt7 cell line displayed detectable telomerase activity, aberrations in *p53*, and in *p21* and *p16* gene expression reported in other immortal breast cell lines of normal or cancerous origin (23–25). Strikingly, in the ENUt4 line, also derived from the treatment of mammary epithelial cells from the same individual with the same chemical carcinogen, dramatic early changes in gene expression were not apparent. However, the possibility of nonfunctional *p21* and/or *p16* gene product have not been ruled out by our studies reported here with this cell line. Such variability specifically for this set of genes is indeed characteristic of immortalized cell lines and breast tumors (23–26). Similarly, for telomerase induction, the most commonly observed mechanism of telomere maintenance in human tumor epithelial cell lines, ENUt4 and ENUt7, displayed a temporal gap encompassing >50 population doublings.

A major goal in the derivation of ENU-immortalized lines was their rapid application toward gaining insights regarding genetic deletions, which occur early during breast tumorigenesis *in vivo*. The detection of a common genetic signature underlying the immortalization of ENUt4 and ENUt7 was largely facilitated and expedited by a genome-wide deletion analysis of early passage ENU-exposed cells. The chronological order of specific genetic losses showed consistent similarities in the two independently isolated cell lines, and most importantly at specific loci, it closely matched early sites of LOH in cancerous breast tissue. Although we have demonstrated the common genetic features of two lines from the same cell strain, repeated attempts for the derivation of additional immortalized cell lines from this or other reduction mammoplasty samples have not yet been made. Deletions detected before the Hayflick limit of cell replication (27), which were common to both ENUt4 and ENUt7, included LOH at loci on the long arm of chromosome 6, encompassing 6q24 to 6q27.

In breast cancer, chromosome 6q21-qter is one of the most frequently deleted regions (28). The candidate region, 6q24-25, harbors a potential gene known to inhibit tumor cell proliferation. *hZAC* (29), *LOT* (30), and *PLAGL1* (31) refer to the same candidate tumor suppressor gene isolated independently and localized to the 6q24-25 region. Down-regulation of *ZAC* is associated with spontaneous trans-

formation in rat ovarian epithelial cells (32). The gene encodes a zinc finger protein widely expressed in human tissues, including the mammary gland (33). *hZAC* is frequently inactivated in primary breast tumors, as well as in breast cancer cell lines and continually passaged cultures of normal human mammary epithelial cells (34). In *hZAC*-inactivated cancer cell lines, ectopic gene expression induces apoptosis and G_1 arrest (29). Additional support for the presence of one or more immortalization-related genes in the 6q21-qter region is also demonstrated by the induction of senescence upon microcell transfer of 6q26-27 into somatic cell hybrids (35). SV40-immortalized human fibroblasts derived from multiple tissues and individuals display a common region of loss at 6q26-27, potentially harboring a senescence-mediating locus, *SEN6* (36). *In vitro* induction of senescence by the *RNASE6PL* gene located at 6q27, a member of a highly conserved family of cytoplasmic RNAses, implicates it as a candidate gene in the SEN6 region (37).

In view of the previously known association of *ZAC* and *SEN6* with immortal transformation of mammalian cell cultures, the finding of LOH encompassing these chromosomal sites in ENU-immortalized cells is not entirely unexpected. To be noted, however, is our approach for delineating the temporal order of gene inactivation in the acquisition of immortalization *in vivo*. The observation of chromosomal deletions in the *ZAC* region within morphologically normal TDLU adjacent to carcinoma is the first demonstration to our knowledge of the loss of a potential tumor suppressor gene before the manifestation of histological changes associated with malignancy in sporadic breast cancer patients. Intriguingly, although 6q26-27 deletions have been observed in benign breast tumors (38), but unlike the region encompassing *hZAC*, we have not found loss of the *SEN6* region to be associated with morphologically normal TDLU within cancerous breast tissue. The absence of *p53* aberrations in normal TDLU adjacent to carcinoma is in agreement with other reports of the relatively late appearance of *p53* aberrations in sporadic breast tumors (39), supporting our view that allelic losses encountered before *p53* mutations *in vitro* and *in vivo* were most likely *p53* independent.

Whereas biallelic or complete inactivation is generally observed for genes targeted by frequent LOH in tumor cells, it is possible that for some genes, deletion of a single allele could have functional consequences preceding the histological changes characteristic of neoplasia. Similar to the causal role of haploinsufficiency reported for the tumor suppressor genes, *PTEN* and *p53*, in promoting tumorigenesis (40, 41), it is conceivable that LOH-based reduction in the dosage of *ZAC* and/or other genes at 6q24 in morphologically normal TDLU may suffice to impair the regulation of proliferative arrest of breast epithelium during normal cycles of growth and differentiation. Deletions at another chromosome site, 3p24, which were unique to the ENUt7 cell line, have been consistently observed in morphologically normal TDLU adjacent to carcinoma (Refs. 10, 11; data shown here). This LOH site harbors the *TRβ1* gene, a common target of allelic loss (11), as well as epigenetic inactivation in breast cancer cells (42). Similarly, LOH at 11p15.5 in ENUt7 cells, also reported in benzo(*a*)pyrene-immortalized human breast epithelial cells (43) may represent partial inactivation of the cyclin-dependent kinase inhibitor, $p57^{KIP2}$.

The consistent lack of early detectable alterations, other than LOH in the 6q24-27 region in ENUt4 cells, albeit surprising, suggests an initiating role for this deletion leading to the immortal phenotype of these cells. It can be speculated that to some degree mechanisms underlying the apparent differences in the two isogenic ENU-immortalized cell lines may reflect factors related to the genesis of genetically and histologically distinct multifocal and/or bilateral breast tumors in the same patient. Such differences could also be a determining predictive factor in downstream progression and cell behavior, particularly in terms of response to potential cancer therapeutic strategies. In conclusion, the ENU-immortalized cell lines described in this report have served reliably to recapitulate early immortalization-associated events *in vivo*. Despite vast differences in the microenvironmental milieu of normal TDLU within tumor tissue and that of ENU-immortalized cells in culture, the close parallel in early allelic losses observed between these entities suggests that cells harboring such alterations may be autonomous of certain types of microenvironmental growth regulatory signals. In this capacity, this new model system could contribute toward an improved understanding of the growth arresting circuitry, which goes awry in progressively aging epithelial cells, often resulting in the initiation and progression of malignancy.

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REFERENCES

1. Smith, H. S., Lu, Y., Deng, G., Martinez, O., Krams, S., Ljung, B. M., Thor, A., and Lagios, M. Molecular aspects of early stages of breast cancer progression. J. Cell. Biochem. Suppl., *17G:* 144–152, 1993.
2. Briand, P., Petersen, O. W., and Van Deurs, B. A new diploid nontumorigenic human breast epithelial cell line isolated and propagated in chemically defined medium. In Vitro Cell. Dev. Biol., *23:* 181–188, 1987.
3. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line. MCF-10. Cancer Res., *50:* 6075–6086, 1990.
4. Stampfer, M. R., and Bartley, J. C. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo(*a*)pyrene. Proc. Natl. Acad. Sci. USA, *82:* 2394–2398, 1985.
5. Wang, F., Weaver, V. M., Petersen, O. W., Larabell, C. A., Dedhar, S., Briand, P., Lupu, R., and Bissell, M. J. Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc. Natl. Acad. Sci. USA, *95:* 14821–14826, 1998.
6. Briand, P., and Lykkesfeldt, A. E. An *in vitro* model of human breast carcinogenesis: epigenetic aspects. Breast Cancer Res. Treat., *65:* 179–187, 2001.
7. Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Eliason, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H., and Miller, F. R. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res. Treat., *65:* 101–110, 2001.
8. Stampfer, M. R., Bodnar, A., Garbe, J., Wong, M., Pan, A., Villeponteau, B., and Yaswen, P. Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. Mol. Biol. Cell, *8:* 2391–2405, 1997.
9. Stampfer, M. R., Garbe, J., Levine, G., Lichtsteiner, S., Vasserot, A. P., and Yaswen, P. Expression of the telomerase catalytic subunit, hTERT, induces resistance to transforming growth factor β growth inhibition in p16INK4A (-) human mammary epithelial cells. Proc. Natl. Acad. Sci. USA, *98:* 4498–4503, 2001.
10. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Wash. DC), *274:* 2057–2059, 1996.
11. Li, Z., Moore, D. H., Meng, Z., Ljung, B-M., Gray, J. W., and Dairkee, S. H. Increased risk of local recurrence is associated with allelic loss in normal lobules of breast cancer patients. Cancer Res., *62:* 1000–1003, 2002.
12. Stampfer, M. R., and Bartley, J. C. Human mammary epithelial cells in culture: differentiation and transformation. *In:* M. E. Lippman and R. B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology, pp. 1–24. Boston: Kluwer Academic Publishers, 1988.
13. Dairkee, S. H., Blayney, C., Smith, H. S., and Hackett, A. J. Monoclonal antibody that defines human myoepithelium. Proc. Natl. Acad. Sci. USA, *82:* 7409–7413, 1985.
14. Dairkee, S. H., Paulo, E. C., Traquina, P., Moore, D. H., Ljung, B-M., and Smith, H. S. Partial enzymatic degradation of stroma allows enrichment and expansion of primary breast tumor cells. Cancer Res., *57:* 1590–1596, 1997.
15. Dairkee, S. H., Deng, G., Stampfer, M. R., Waldman, F. M., and Smith, H. S. Selective cell culture of primary breast carcinoma. Cancer Res., *55:* 2516–2519, 1995.
16. Vesselinovitch, S. D., Rao, K. V., Mihailovich, N., Rice, J. M., and Lombard, L. S. Development of broad spectrum of tumors by ethylnitrosourea in mice and the modifying role of age, sex, and strain. Cancer Res., *34:* 2530–2538, 1974.
17. Dairkee, S. H., and Heid, H. Cytokeratin profile of immunomagnetically separated epithelial subsets of the human mammary gland. In Vitro Cell. Dev. Biol., *29:* 427–432, 1993.
18. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhutz, A. J. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature (Lond.), *396:* 84–88, 1998.

19. Kerangueven, F., Noguchi, T., Coulier, F., Allione, F., Wargniez, V., Simony-Lafontaine, J., Longy, M., Jacquemier, J., Sobol, H., Eisinger, F., and Birnbaum, D. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. Cancer Res., *57:* 5469–5474, 1997.
20. Osborne, R. J., and Hamshere, M. G. A genome-wide map showing common regions of loss of heterozygosity/allelic imbalance in breast cancer. Cancer Res., *60:* 3706–3712, 2000.
21. Shen, C-Y., Yu, J-C., Lo, Y-L., Kuo, C-H., Yue, C-T., Jou, Y-S., Huang, C-S., Lung, J-C., and Wu, C-W. Genome-wide search for loss of heterozygosity using laser capture microdissected tissue of breast carcinoma: an implication for mutator phenotype and breast cancer pathogenesis. Cancer Res. *60:* 3884–3892, 2000.
22. O'Connell, P., Pekkel, V., Fuqua, S. A., Osborne, C. K., Clark, G. M., and Allred, D. C. Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. J. Natl. Cancer Inst. (Bethesda), *90:* 697–703, 1998.
23. Villadsen, R., Nielsen, K. V., Bolund, L., and Briand, P. Complete loss of wild-type TP53 in a nontransformed human epithelial cell line is preceded by a phase during which a heterozygous TP53 mutant effectively outgrows the homozygous wild-type cells. Cancer Genet. Cytogenet., *116:* 28–34, 2000.
24. Musgrove, E. A., Lilischkis, R., Cornish, A. L., Lee, C. S., Setlur, V., Seshadri, R., and Sutherland, R. L. Expression of the cyclin-dependent kinase inhibitors p16INK4, p15INK4B, and p21WAF1/CIP1 in human breast cancer. Int. J. Cancer, *63:* 584–591, 1995.
25. Bisogna, M., Calvano, J. E., Ho, G. H., Orlow, I., Cordon-Cardo, C., Borgen, P. I., and Van Zee, K. J. Molecular analysis of the INK4A and INK4B gene loci in human breast cancer cell lines and primary carcinomas. Cancer Genet. Cytogenet., *125:* 131–138, 2001.
26. Milde-Langosch, K., Bamberger, A. M., Rieck, G., Kelp, B., and Loning, T. Overexpression of the p16 cell cycle inhibitor in breast cancer is associated with a more malignant phenotype. Breast Cancer Res. Treat., *67:* 61–70, 2001.
27. Hayflick, L. Mortality and immortality at the cellular level. A review. Biochemistry, *62:* 1180–1190, 1997.
28. Mertens, F., Johansson, B., Hoglund, M., and Mitelman, F. Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. Cancer Res., *57:* 2765–2780, 1997.
29. Varrault, A., Ciani, E., Apiou, F., Bilanges, B., Hoffmann, A., Pantaloni, C., Bockaert, J., Spengler, D., and Journot, L. hZAC encodes a zinc finger protein with antiproliferative properties and maps to a chromosomal region frequently lost in cancer. Proc. Natl. Acad. Sci. USA, *95:* 8835–8840, 1998.
30. Abdollahi, A., Roberts, D., Godwin, A. K., Schultz, D. C., Sonoda, G., Testa, J. R., and Hamilton, T. C. Identification of a zinc-finger gene at 6q25: a chromosomal region implicated in development of many solid tumors. Oncogene, *14:* 1973–1979, 1997.
31. Kas, K., Voz, M. L., Hensen, K., Meyen, E., and Van de Ven, W. J. Transcriptional activation capacity of the novel PLAG family of zinc finger proteins. J. Biol. Chem., *273:* 23026–23032, 1998.
32. Abdollahi, A., Godwin, A. K., Miller, P. D., Getts, L. A., Schultz, D. C., Taguchi, T., Testa, J. R., and Hamilton, T. C. Identification of a gene containing zinc-finger motifs based on lost expression in malignantly transformed rat ovarian surface epithelial cells. Cancer Res., *57:* 2029–2034, 1997.
33. Spengler, D., Villalba, M., Hoffmann, A., Pantaloni, C., Houssami, S., Bockaert, J., and Journot, L. Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. EMBO J., *16:* 2814–2825, 1997.
34. Bilanges, B., Varrault, A., Basyuk, E., Rodriguez, C., Mazumdar, A., Pantaloni, C., Bockaert, J., Theillet, C., Spengler, D., and Journot, L. Loss of expression of the candidate tumor suppressor gene ZAC in breast cancer cell lines and primary tumors. Oncogene, *18:* 3979–3988, 1999.
35. Sandhu, A. K., Hubbard, K., Kaur, G. P., Jha, K. K., Ozer, H. L., and Athwal, R. S. Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6. Proc. Natl. Acad. Sci. USA, *91:* 5498–5502, 1994.
36. Banga, S. S., Kim, S., Hubbard, K., Dasgupta, T., Jha, K. K., Patsalis, P., Hauptschein, R., Gamberi, B., Dalla-Favera, R., Kraemer, P., and Ozer, H. L. SEN6, a locus for SV40-mediated immortalization of human cells, maps to 6q26–27. Oncogene, *14:* 313–321, 1997.
37. Acquati, F., Morelli, C., Cinquetti, R., Bianchi, M. G., Porrini, D., Varesco, L., Gismondi, V., Rocchetti, R., Talevi, S., Possati, L., Magnanini, C., Tibiletti, M. G., Bernasconi, B., Daidone, M. G., Shridhar, V., Smith, D. I., Negrini, M., Barbanti-Brodano, G., and Taramelli, R. Cloning and characterization of a senescence inducing and class II tumor suppressor gene in ovarian carcinoma at chromosome region 6q27. Oncogene, *20:* 980–988, 2001.
38. Tibiletti, M. G., Sessa, F., Bernasconi, B., Cerutti, R., Broggi, B., Furlan, D., Acquati, F., Bianchi, M., Russo, A., Capella, C., and Taramelli, R. A large 6q deletion is a common cytogenetic alteration in fibroadenomas, pre-malignant lesions, and carcinomas of the breast. Clin. Cancer Res., *6:* 1422–1431, 2000.
39. Franco, N., Picard, S. F., Mege, F., Arnould, L., and Lizard-Nacol, S. Absence of genetic abnormalities in fibroadenomas of the breast determined at *p53* gene mutations and microsatellite alterations. Cancer Res., *61:* 7955–7958, 2001.
40. Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. Pten is essential for embryonic development and tumor suppression. Nat. Genet., *19:* 348–355, 1998.
41. Venkatachalam, S., Shi, Y. P., Jones, S. N., Vogel, H., Bradley, A., Pinkel, D., and Donehower, L. A. Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. EMBO J., *17:* 4657–4667, 1998.
42. Li, Z., Meng, Z., Chandrasekaran, R., Kuo, W-L., Collins, C. C., Gray, J. W., and Dairkee, S. H. Biallelic inactivation of the *thyroid hormone receptor β1* gene in early stage breast cancer. Cancer Res., *62:* 1939–1943, 2002.
43. Nijjar, T., Wigington, D., Garbe, J. C., Waha, A., Stampfer, M. R., and Yaswen, P. $p57^{KIP2}$ Expression and loss of heterozygosity during immortal conversion of cultured human mammary epithelial cells. Cancer Res., *59:* 5112–5118, 1999.

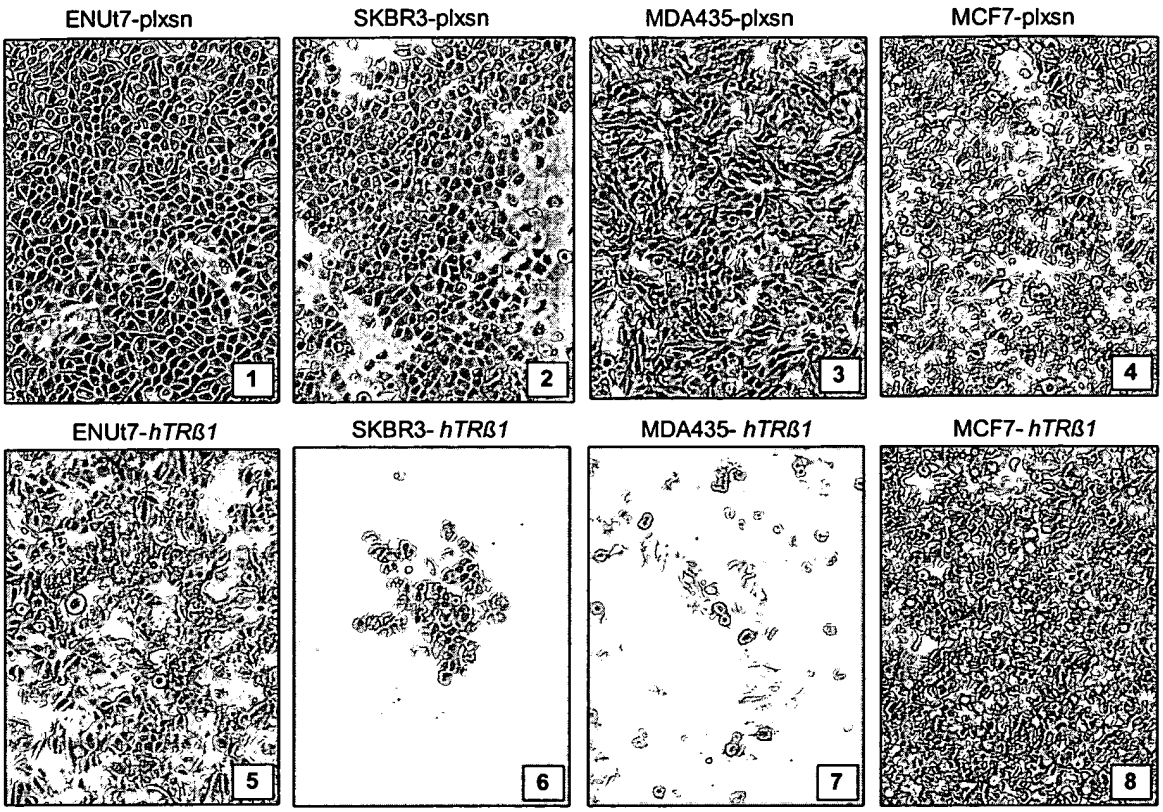

Figure 2 – Effects of *hTRβ1* gene on growth and morphology of human breast epithelial cells. Panels 1-4 represent control cultures in which an empty vector (plxsn) lacking the *hTRβ1* gene was introduced into cells. Panels 5-8 represent introduction of the *hTRβ1* gene-containing vector into the same cell cultures. The non-malignant cell line, ENUt7 in panels 1, and 5, and the breast cancer cell lines SKBR3, and MDA435 (panels 2,3,6,7) display the most significant effects of growth reduction by the *hTRβ1* gene. In contrast, the MCF7 cell line (panels 4,8) does not display any growth inhibitory effects. Brightfield micrographs were made at 200X magnification using an inverted microscope.

Cell cycle analysis of *hTRß1* - induced growth arrest

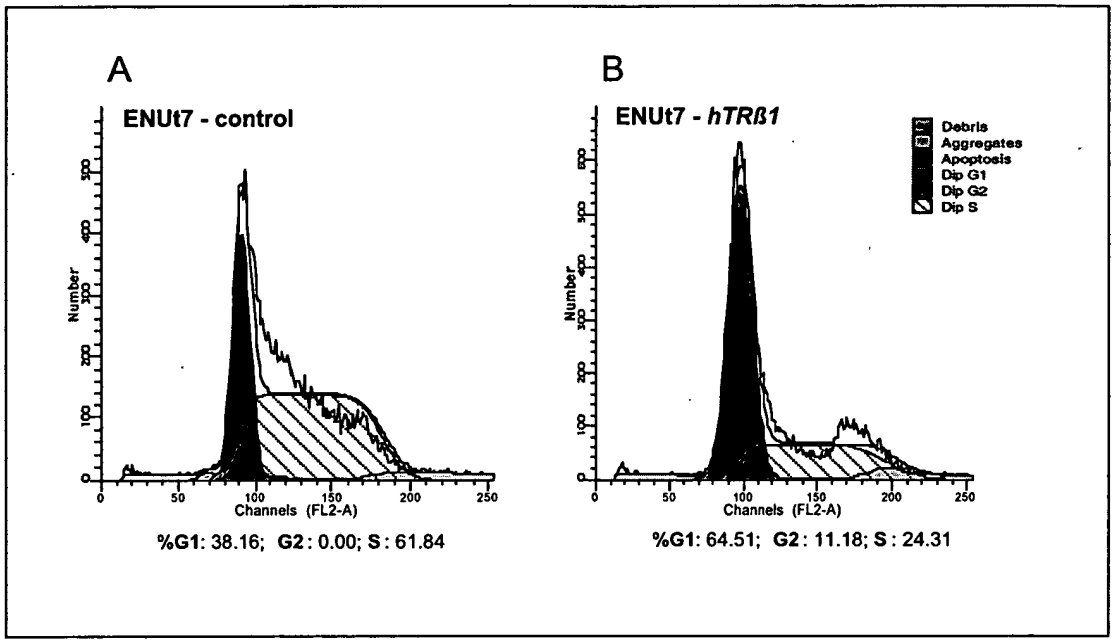

Figure 3 - *hTRβ1* induced growth inhibition in the non-malignant epithelial cell line, ENUt7 measured by FACS analysis of the cell cycle. Panel A represents analysis of the empty vector (plxsn) transduced control cells. Panel B represents data from *hTRβ1* gene transduced cultures of the same cells. While the untransduced cells displays the majority of the culture in the proliferative S phase of the cell cycle, the *hTRβ1* transduced culture consists of a reduction in such cells to less than 25%. Concurrently, cells appear to be accumulating in the G1 and G2 phase of the cell cycle.

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